

THE ROLE OF ASCORBIC ACID IN ZINC OXIDE NANOPARTICLES INDUCED LUNG TOXICITY IN ADULT MALE ALBINO RATS

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ABSTRACT

Zinc oxide nanoparticles (ZnONPs) have been widely used in consumer and industrial products, especially in cosmetics, food additives, photo-electricity and rubber industry. The aim of the work was to evaluate toxic effect of 30 days administration of ZnONPs on lung and the ameliorative role of ascorbic acid in adult male albino rats. They received orally 400mg/kg/day of ZnONPs for 30 days and compared with others received the same dose of ZnONPs + 20mg/kg of ascorbic acid regarding GSH, NO, DNA damage and histopathological changes of the lung. Oral administration of 1/20 LD₅₀ of ZnONPs for 30 days led to decrease in serum GSH, increased levels of NO, DNA damage detected by comet assay and histopathological changes in the lung in the form of collapsed alveoli, destruction of interalveolar septa with alveolar dilatation, thick interalveolar septa, heavy infiltration of inflammatory cells and inflammatory exudate. Ascorbic acid administration along with ZnONPs led to attenuation of oxidative stress and pulmonary toxicity induced by ZnONPs, but there was nonsignificant improvement in DNA damage proved by Comet assay. We concluded that ascorbic acid administration along with ZnONPs led to attenuation of oxidative stress and pulmonary toxicity induced by ZnONPs, but there was nonsignificant improvement in DNA damage proved by Comet assay.

Keywords: zinc oxide; nanoparticles; lung; ascorbic acid; toxicity

INTRODUCTION

Zinc oxide nanoparticles (ZnONPs) are new type of high-functional fine inorganic materials having one dimension that measures 100 nanometers or less (Osmond and McCall, 2010 & Elsamad et al., 2014). They have been widely used in cosmetics, sun screens, foot-care ointments, antimicrobials, food additives, cancer therapy, over the counter topical products (mouth washes and anti-dandruff shampoos), fungicide, in paints, in photo electricity and rubber industry (Guan et al., 2012).

Oral exposure can occur directly from food, water or orally administered drugs. (Osmond and McCall, 2010). After gut absorption, they are transported to the blood causing adverse biological reactions in several organs. The major sites of interaction with ZnONPs are lung, liver, kidneys and heart (Johnston et al., 2010). The acidic environment of the lung lining fluid result in ZnONPs dissolution, leading to transient increases in the concentration of Zn⁺² ions and local pulmonary toxicity (Vandebriel and De jong, 2012).

Several studies suggested that

toxicity of ZnONPs is attributed to disruption of cellular Zinc homeostasis by dissolution of ZnONPs with increased levels of these ions, production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) which lead to oxidative stress, inflammation, DNA damage and cell death (Muserrat et al., 2009; George et al., 2010 & Kao et al., 2012).

Ascorbic acid (AA) is a naturally occurring organic compound with antioxidant properties attributed to direct radical scavenging, trapping of reactive oxygen species and metal chelation causing reduction in oxidative damage, DNA damage and inflammation (Arrigoni and De Tulio, 2002)

Several invivo and invitro studies proved that AA is a useful agent for attenuating the lung injury caused by increased oxidative stress, preventing protein damage, cytotoxicity and apoptosis (Nemenqani, 2015).

The aim of this study was to investigate the toxicity of administration of ZnONPs for 30 days on the lung and the role of Ascorbic acid in amelioration of this toxic effect. For this purpose Biochemical parameters for oxidative stress (reduced glutathione and Nitric oxide) were analyzed, lung damage was histopathologically examined and DNA affection was evaluated by Comet assay

MATERIALS AND METHODS

1. Chemicals: White odorless fine powder of **Zinc oxide nanoparticles (ZnONPs)** manufactured by **Sigma-Aldrich chemical company, USA** were purchased from **Sigma-Egypt (Eltayaran st., Nasr city-Cairo)**. Its CAS No is 1314-13-2,

with particle size <100nm and molecular weight: 81.39 g/mol. Also white odorless powder (100mg) of pure **ascorbic acid** were obtained from **Elgomhoria Pharmaceuticles Co (Elsawaf st., Zagazig, Egypt)**.

2. Animals: fifty adult male albino rats weighing between 180-220 g for each, with average age 60-70 days were used in this study. They were obtained from Animal house of the Faculty of Medicine, Zagazig University. The study was performed at Animal house of the Faculty of medicine, Zagazig University. All animals received human care in compliance with the animal guidelines and ethical regulations in accordance with "The Guide for The Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources et al., 1996).

all animals were left to acclimatize 14 days prior to the experiment and were placed in plastic cages free from any source of chemical contamination under controlled conditions with an ambient range of temperature (22±2°c), relative humidity 50±5% and a 12 h light-cycle with free access to tap water and balanced food rich in all stuffs important to maintain their health before and during drug administration. Rats were divided into five groups as follow:

Group I:- consisted of 20 rats subdivided equally and randomly into:-

Subgroup Ia (negative control) (n=10): each rat received regular diet and tap water for 30 days to measure the basic parameters

Subgroup Ib (positive control) (n=10): animals received 1ml/kg/day distilled water (solvent of zinc oxide and ascorbic acid) orally by gavage for 30 days.

Group II (Ascorbic acid group)

(AA): (n=10) rats received orally 20mg/kg/day ascorbic acid dissolved in 1 ml distilled water for 30 days (**Devi and Iatha, 2011**).

Group III (Zinc oxide nanoparticles treated group (ZnONPs): (n=10):- rats were treated with (400mg/kg/day) of Zinc oxide nanoparticles dissolved in 1 ml distilled water orally by gavage for 30 days. This dose represents 1/20 of the oral LD50 of ZnONPs (8000mg/kg) (**NIOSH, 2010**).

Group IV (ZnONPs + Ascorbic acid treated group (ZnONPs+AA): n=10):- rats were treated with (400mg/kg/day) ZnONPs along with ascorbic acid (20mg/kg/day) both dissolved in 1 ml distilled water, gavaged once daily for 30 days.

3. Biochemical analysis:

At the end of the experiment; rats were anaesthetized with ether then venous blood samples were collected from the retro-orbital plexuses by means of micro capillary glass tubes under light ether anesthesia (**Johnson, 2007**). Blood samples were centrifuged to separate the serum and maintained at (-20°C) to be used for estimation of reduced glutathione (GSH) by the method of **Moron et al. (1979)** and nitric oxide (NO). according to the method of **Montgomery and Dymock, (1961)**.

4. Histopathological examination and comet assay:

After blood samples collection rats were sacrificed and the lungs were removed and grossly inspected to assess any gross abnormalities, then

divided into two parts: the first part was fixed in 10% formaline for histopathological examination by light microscope in Histology Department Faculty of Medicine, Zagazig University, according to (**Bancroft and Gamble, 2002 & Kiernan, 2001**), the second part was put in saline for Comet assay to investigate DNA damage according to the method of **Singh et al. (1988) & Khan et al. (2015)** in Animal Reproductive Research Institute (ARRI) of Agricultural Research Centre of Ministry of Agriculture and Land Reclamation (**Elharam, Giza**)

3. Statistical analysis: All results were expressed as mean±standard deviation (SD). For statistical analysis, values among groups were analyzed by one-way analysis of variance (ANOVA). Comparison between experimental groups and each other was made by Least significant difference (LSD).

RESULTS

1. Biochemical results:

Non significant differences regarding mean values of serum GSH and NO among negative control (Ia) (10.88±0.69, 42.43 ± 5.44), positive control (Ib) (10.83 ± 0.72, 43.75 ± 6.11) and ascorbic acid groups (II) (11.01 ± 0.75, 44.26 ± 6.75) respectively were detected (P>0.05) by ANOVA test, so we used negative control group as a standard reference for comparison with other treated groups (table 1).

Table (1): Statistical Comparison of mean values of biochemical parameters (serum reduced glutathione (mmol/dl), nitric oxide($\mu\text{mol/l}$)and Comet test analysis(comet tail length (μm), Tail DNA %, Unit tail moment) in negative control (-ve), positive control (+ve) and ascorbic acid (A.A.) groups

Parameter		Group	-ve control group N=10	+ve control group N=10	Ascorbic acid group N=10	F	P
		Mean \pm SD					
Biochemical parameter	GSH(mmol/dl)		10.88 \pm 0.69	10.83 \pm 0.72	11.01 \pm 0.75	0.166	0.848 NS
	NO($\mu\text{mol/l}$)		42.43 \pm 5.44	43.75 \pm 6.11	44.26 \pm 6.75	0.238	0.790 NS
Comet test	Tail length(μm)		1.68 \pm 0.26	1.65 \pm 0.25	1.69 \pm 0.27	0.064	0.938 NS
	Tail DNA%		1.57 \pm 0.26	1.61 \pm 0.26	1.61 \pm 0.27	0.404	0.235 NS
	Unit tail moment		15.47 \pm 1.5	15.12 \pm 1.5	15.55 \pm 1.6	0.222	0.802 NS

N= number of rats SD: Standard Deviation GSH:reduced glutathione mmol/dl: millimoles per deciliter. NO: Nitric oxide $\mu\text{mol/l}$: micromole per liter μm =micrometers *P: Analysis of variance test (ANOVA) NS: nonsignificant

Significant differences among -ve control, ZnONPs and ZnONPs+AA groups regarding mean values of GSH &NO were detected by ANOVA test ($P<0.001$)(Table 2).Least significant difference test (LSD) showed significant decrease in the mean values of GSH in ZnONPs when compared with -ve control and ZnONPs+AA groups($^aP<0.001$) while no significant difference in the mean values of GSH

in ZnONPs+AA when compared with control group ($^bP>0.05$). Regarding mean values of NO, there were significant increase in ZnONPs group when compared with -ve control and ZnONPs+AA groups($^cP<0.001$)while no significant difference in the mean values of GSH in ZnONPs+AA when compared with control group($^dP>0.05$)(Table 2).

Table (2): Statistical Comparison of mean values of biochemical parameters (serum reduced glutathione (mmol/dl), nitric oxide($\mu\text{mol/l}$)and Comet test analysis(comet tail length (μm), Tail DNA%, Unit tail moment) in different studied groups.

Group Parameter		-ve Control (N= 10)	Zinc Oxide NPs (N= 10)	Zinc Oxide NPs + Ascorbic acid (N= 10)	F	p
		Mean \pm SD				
Biochemical parameters	GSH: (mmol/dl)	10.88 \pm 1.69	1.46 \pm 0.64 ^a	8.2 \pm 1.02 ^b	164.1235	<0.001*
	NO:($\mu\text{mol/l}$)	42.43 \pm 5.44	114.26 \pm 12.44 ^c	41.02 \pm 8.12 ^d	210.2758	<0.001*
Comet test	Comet tail length (μm)	1.68 \pm 0.26	4.07 \pm 0.10	3.74 \pm 0.07	609.9758	<0.001*
	Tail DNA %	1.57 \pm 0.25	3.80 \pm 0.13	3.48 \pm 0.04	538.4815	<0.001*
	Unit tail moment	2.68 \pm 0.12 ^e	15.47 \pm 1.3	12.99 \pm 1.4 ^f	378.0827	<0.001*

NPs:nanoparticles N= number of rats SD: Standard Deviation -ve:negative ZnONPs: zincoxide nanoparticles AA: ascorbic acid GSH:reduced glutathione mmol/dl: millimoles per deciliter. NO: Nitric oxide $\mu\text{mol/l}$: micromole per liter μm =micrometers *P: Analysis of variance test(ANOVA). Least significant difference (Lsd); ^aP<0.001vs -ve control & ZnNP+AA groups, ^bP >0.05 vs control group. ^cP<0.001 vs -ve control& ZnNP+AA groups, ^dP>0.05 vs -ve control group, ^eP<0.001vs ZnONPs and ZnNP+AA, ^fP>0.05 vs ZnONPs group

2. Single cell gel electrophoresis (Comet assay):

No significant differences regarding mean values of comet tail length, percentage of tail DNA (tail DNA%) and tail moment among -ve, +ve controls and Ascorbic acid groups ($p>0.05$) by ANOVA test, so we used negative control group as a standard reference for comparison with other treated groups(Table1). Table (2) showed significant difference among -ve control, ZnONPs and ZnONPs+AA regarding comet tail length (μm), tail DNA% and unit tail moment ($P<0.001$)

by ANOVA test. Least significant test revealed significant increase in unit tail moment in both ZnONPs and ZnONPs+AA groups when compared with control group (^e $p<0.001$) while no significant difference in ZnONPs+AA when compared with ZnONPs was detected(^f $P>0.05$). **Figure a** showed normal lung nuclei and undamaged cells in control group (fig1,2a) while abnormal tailed nuclei & damaged cells in ZnONPs group(fig3,4 a) and ZnONPs+AA groups(fig 5,6a) were detected.

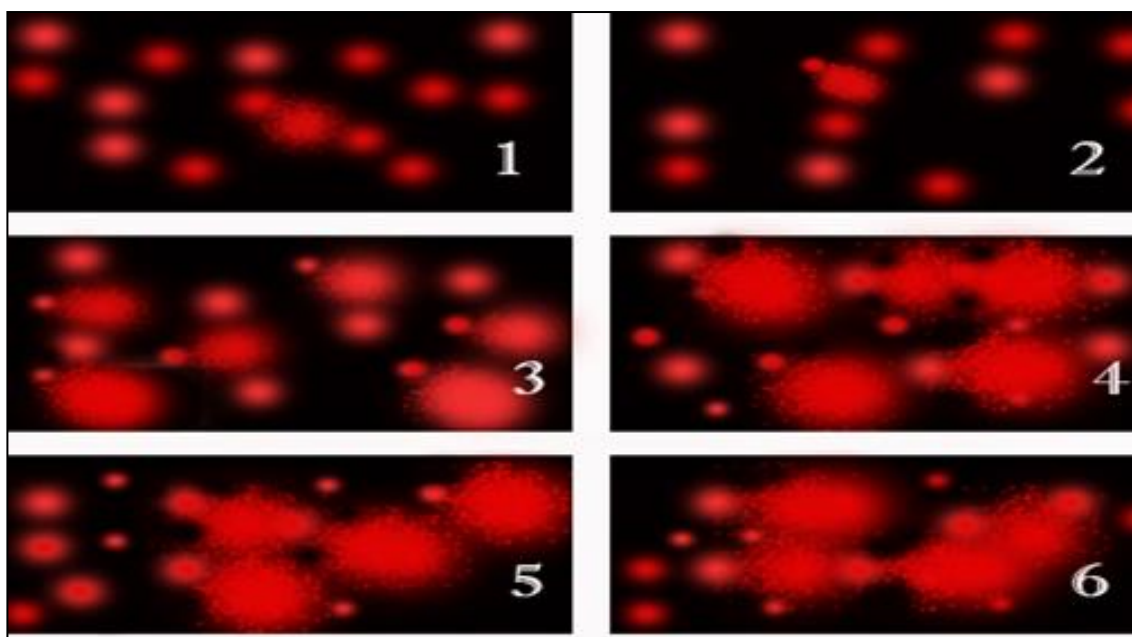


Figure (a): Nuclei of lung cells of male rats showing almost normal condensed type nuclei & undamaged cells in control group (1,2). Abnormal tailed nuclei & damaged cells of ZnONPs treated group (3,4), abnormal tailed nuclei & damaged cells in ZnONPs+AA group (5,6)

3. Histopathological results: The light microscopical examination of hematoxylin and eosin (H&E) stained sections from the lung of control and ascorbic acid groups, showed normal spongy histological appearance with numerous alveoli connected together with alveolar pores and opens into alveolar sacs, thin inter alveolar septa, bronchioles and blood vessels (Fig. b,c).while diffuse alveolar damage with marked consolidation of lung tissue, collapsed alveoli, marked thickening of interalveolar septa and extravasation of

RBCs in the alveolar lumen, hyperemic foci, thickened wall pulmonary blood vessels, heavy infiltration with chronic inflammatory cells mainly lymphocytes and partial shedding of mucosal lining of bronchioles were detected in ZnONPs intoxicated group (Fig.d,e,f). In ZnONPs+ ascorbic acid treated group lung sections showed normal alveoli with some collapsed alveoli scattered inbetween, mild thickening of interalveolar septa and inflammatory cellular infiltration (Fig. g)

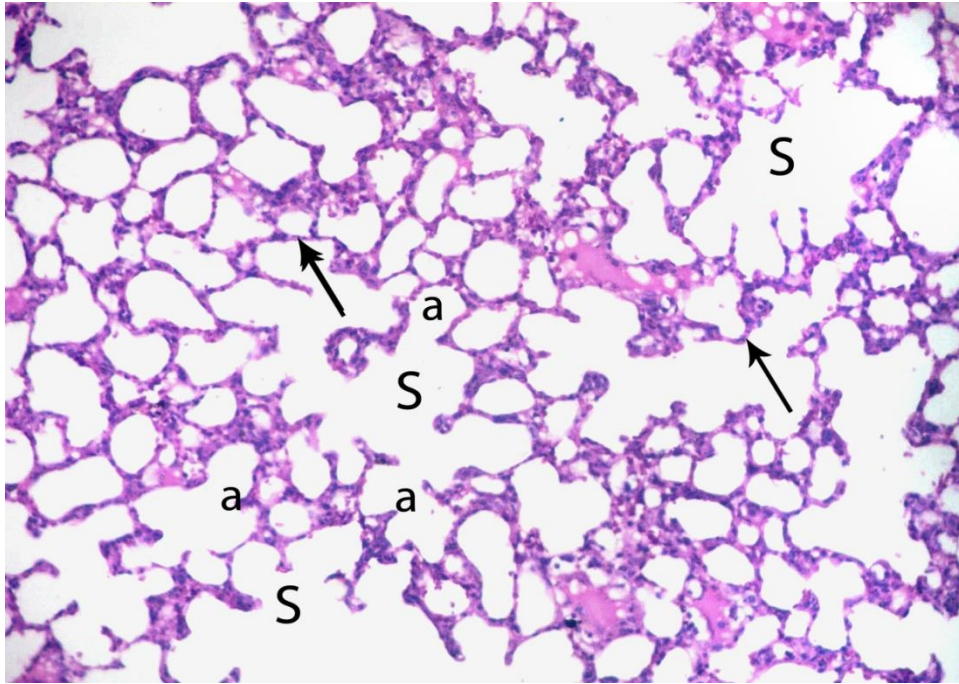


Figure (b): A photomicrograph of section in lung tissue obtained from an adult male albino rat of control group showing: Normal spongy histological appearance with numerous alveoli (a) connected together with alveolar pores and opens into alveolar sacs(s), thin inter alveolar Septa (arrow). (H&E $\times 200$)

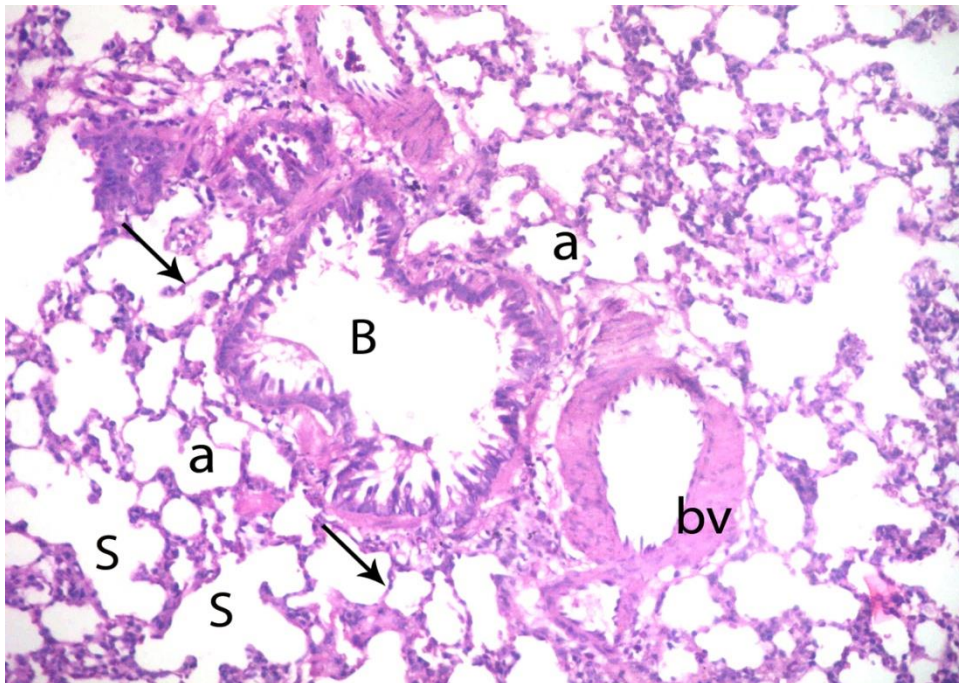
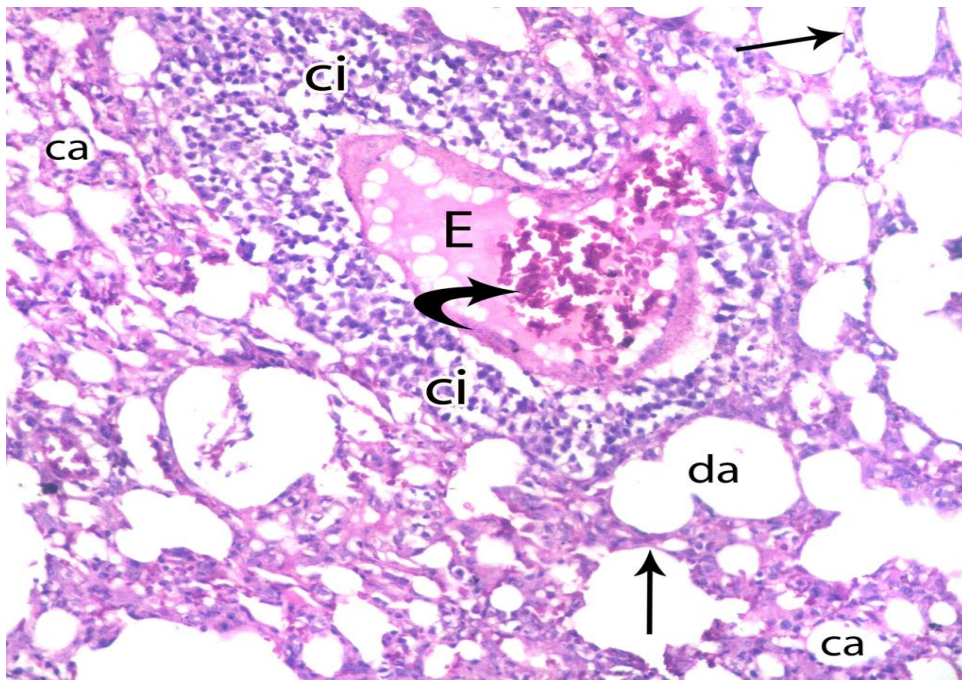
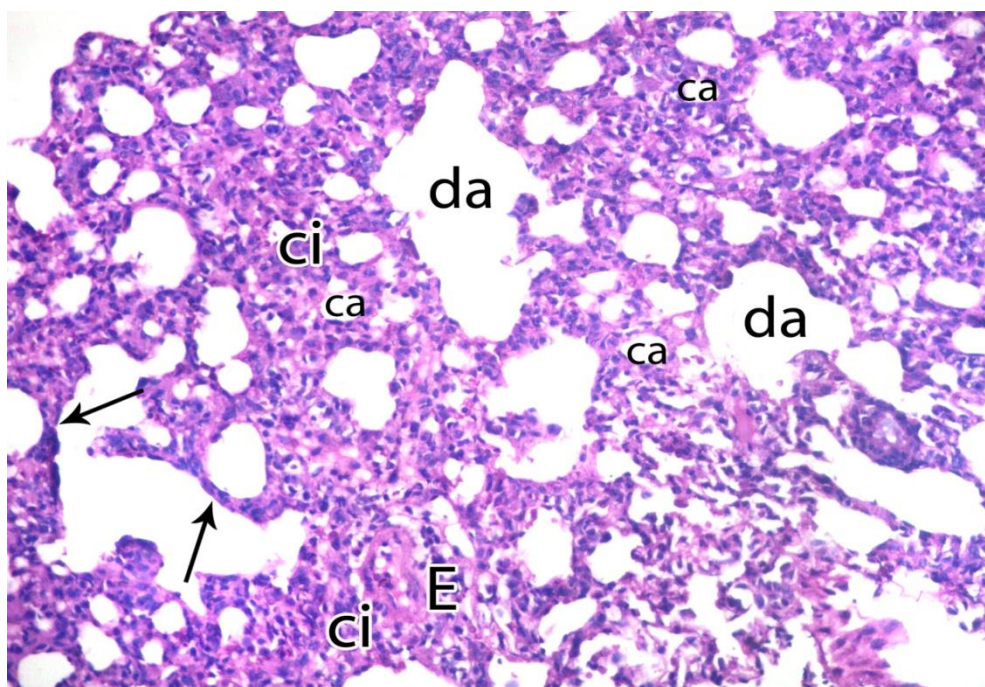


Figure (c): A photomicrograph of section of lung tissue obtained from an adult male albino rat of ascorbic acid group showing: pulmonary alveoli (a) opens into alveolar sacs(s) and thin interalveolar septa (arrow), bronchiole (B) and blood vessel (bv) (H&E $\times 200$).



Figure(d) section in lung tissue obtained from an adult male albino rat of ZNONPs treated group showing: collapsed alveoli(ca), thick interalveolar septa (arrow), destruction of interalveolar septa with alveolar dilatation(da), heavy infiltration of inflammatory cells mainly lymphocytes(ci) with acidophilic exudates (E) and extravasation of RBCs in the alveolar lumen(curved arrow) (H&E $\times 200$).



Figure(e) section in lung tissue obtained from an adult male albino rat of ZnONPs treated group showing: Thick interalveolar septa(arrow), diffuse alveolar damage, marked consolidation of lung tissue with collapsed alveoli(ca) and dilatation of adjacent ones(da) with destruction of interalveolar septa and heavy infiltration with chronic inflammatory cells(ci)and inflammatory exudates (E) (H&E $\times 200$).

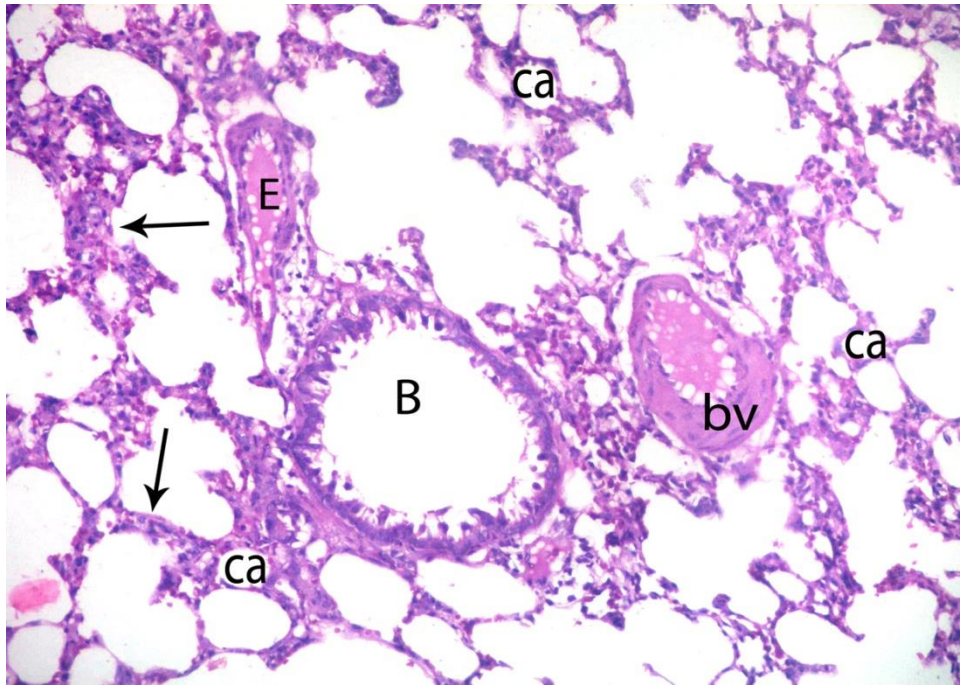


Figure (f): A photomicrograph of section in lung tissue obtained from an adult male albino rat of ZNONPs treated group showing: collapsed alveoli (ca), thickened inter alveolar septa (arrow), bronchiole (B) and thickwall and congested pulmonary blood vessels (bv)and inflammatory exudate (E) (H&E $\times 200$).

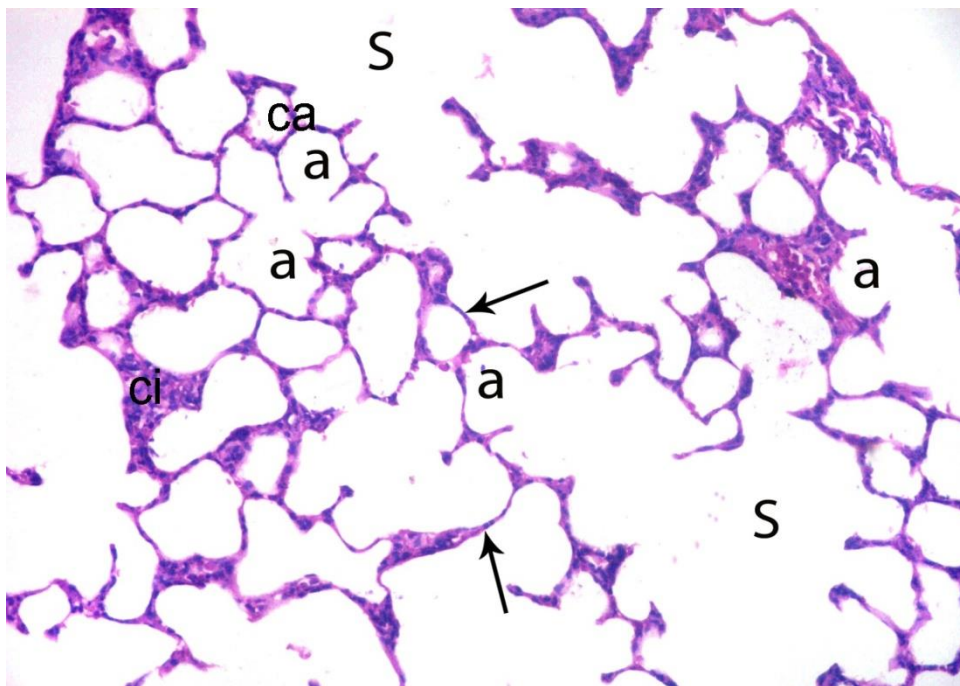


Figure (g): A photomicrograph of section of lung tissue obtained from an adult male albino rat of ZNONPs + AA group showing: Normal alveoli (a) connected together with alveolar pores and opens into alveolar sacs(s). Some collapsed alveoli in between (ca), mild thickening of interalveolar septa (arrow) and inflammatory cellular infiltration (ci) (H&E $\times 200$).

DISCUSSION

Zinc oxide nanoparticles are one of the most utilized nanoparticles. The human body may be intentionally or unintentionally exposed to nanoparticles through oral ingestion, inhalation, dermal exposure and intravenous injection (**Jacobsen et al., 2015** and **Nemenqani, 2015**). **Landsiedel et al. (2014)** reported that nanoparticles are more toxic than larger counter parts as the dissolution rate increase with decrease particle size. So the dissolution of Zn^{+2} ions from the particles is the major determinant for ZnO mediated toxicity and cell death in mice. Earlier study suggested that uptake of dissolved zinc ions and production of reactive oxygen species is the cause for ZnONPs cell wall damage, enhanced membrane permeability, mitochondrial weakness, DNA damage, inflammatory response, cell growth inhibition and cell death (**Sirelkhatem et al., 2015**).

L-ascorbic acid is the most active form of vitamin C. being antioxidant; it acts as free radical scavenger. It can reduce DNA damage and inflammatory response of many toxic substances (**Bakhshiani and Fazilati, 2014**).

The aim of this study was to investigate the toxic effects of 30 days administration of ZnONPs and evaluate the ameliorative role of ascorbic acid in adult male albino rats. For this purpose, the level of serum reduced glutathione (GSH) and serum nitric oxide (No) were measured to evaluate oxidative stress caused by ZnONPs. Histopathological changes in the lung were also evaluated to confirm ZnONPs toxicity. Comet assay was done to study the presence of DNA damage

Administration of 400mg/kg/day of

ZnONPs for 30 days showed significant decrease in serum GSH as a marker of oxidative stress. This observations were in line with the results of **DeBerardis et al. (2010)**, who reported that in vitro studies on human colon carcinoma cell line exposed to 50-70 nm ZnONPs resulted in decreased viability, increased hydrogen peroxide free radicals and decreased glutathione (GSH) levels. This decrease in GSH levels can be due to either direct action of ZnONPs on GSH synthesis **Khan et al. (2015)** or interference with its action in scavenging free radicals (**Ansari et al., 2010**).

However, in contrast to this, **Afifi et al. (2015)** demonstrated that ZnONPs (10mg/kg) has the ability to protect the tissue from oxidative stress induced by diabetes in rats proved by elevation of the decreased levels of GSH. Moreover, **Dkhil et al. (2015)** reported that ZnONPs in a dose 10mg/kg for 5 days are able to prevent the loss of GSH during oxidative damage induced by infection with *Eimeria Papillata* in jejunum of mice. However, this contrast could be explained by their usage of low dose (10mg/kg) and short period of experiment (5 days).

A significant increase in mean values of serum GSH in ZnONPs +Ascorbic acid group when compared with ZnONPs intoxicated group was detected. These results can be supported by, **Nemanqani et al. (2015)** who recorded that oral administration of vitamin c (30mg/kg) along with zinc oxide nano particles resulted in restoration of hepatic GSH levels in male wister rats exposed to 300mg and 1g/kg/day for four weeks.

Also, **Fukui et al. (2015)** illustrated that oral intake of ascorbic

acid can attenuate acute pulmonary oxidative stress and inflammation caused by intra tracheal injection of ZnONPs. Moreover, **Othman and Hussein, (2015)** mentioned that while GSH normally functions to maintain vitamin C and other cellular components in the reduced states, vitamin C can serve as an essential antioxidant in the presence of severe GSH deficiency.

Ascorbic acid is the most abundant hydrophilic antioxidant in the body acts in combating free radicles and protecting bio membranes from oxidative damage induced by pollution (**Hassan and Jassim, 2010 and Skrivan et al., 2012**).

From the previous studies it is clear that ascorbic acid can restore the decreased levels of GSH induced by ZnONPs through suppression of oxidative stress and lipid peroxidation together with neutralization of the excess amounts of free radicals keeping GSH in the reduced state.

The results of present study showed highly significant increase in mean values of serum NO ($p < 0.001$) in ZnONPs group when compared with negative control group.

The results of the present study were in accordance with **Fahmy et al. (2014)** who revealed that there was significant increase in NO levels after exposure to ZnONPs (7 and 35 μ g/ml) in hemolymph and tissues of fresh water snails *Biomphalaria Alexandrina* compared with their corresponding controls after 3 weeks.

High amounts of NO are released from the inducible nitric oxide synthase (iNOS) enzyme isoform in response to inflammatory stimuli from variety of cell types (**Pfeilschifter et al., 1992 and Saura et al., 1995**). Increased

serum nitric oxide levels in ZnONPs intoxicated rats suggest that these nanoparticles can induce oxidative stress and increase pro inflammatory mediators (**Faddah et al., 2012**).

Oxidative stress and the rising levels of free radicals have a strong relation with the increased levels of inducible nitric oxide synthase (iNOS) protein in the lung with production of NO as a compensatory mechanism (**Porter et al., 2006**).

In contrast to our results, **Dawei et al. (2009)** reported that ZnONPs (0.4, 1.6 and 6.4 μ g/ml) are able to protect cell membrane integrity in mice intestinal epithelial cells against oxidative stress damage, increase antioxidant enzyme levels and decrease nitric oxide (NO) and malondialdehyde (MDA) levels.

Furthermore, **Hussein et al. (2014)** concluded that ZnONPs (10mg/kg/day) administration for one month significantly reduces the elevated levels of NO in the serum of diabetic rats.

The results of the present study showed no-significant difference in mean values of serum NO in ZnONPs + AA group when compared with -ve control group, but there was highly significant decrease in mean values of serum NO in ZnONPs + AA group when compared with ZnONPs intoxicated group.

These results coincide with **Fukui et al. (2015)** who reported that AA has a preventive effect against lung inflammation caused by ZnONPs. This was attributed to inhibition of the production of reactive oxygen species (ROS) caused by activation of phagocytes, such as neutrophils and macrophages and suppression of cytokine-induced neutrophil chemoattractant (CINC) genes.

Improvement of oxidative stress was associated with decreased levels of proinflammatory mediators such as NO.

In addition, **Baltalarli et al. (2006)** reported that administration of moderate doses of vitamin C almost completely prevents protein damage, apoptosis and lung injury caused by several toxic materials (

However, in contrast to our results, **Wang et al. (2014)** suggested that vitamin C increase the cytotoxicity of ZnONPs. These results could be attributed to that the acidity of ascorbic acid enhances the release of Zn ions from ZnONPs to an aqueous medium and inducing more serious cytotoxicity by increased levels of zinc ions.

Also, **Abo Zeid et al. (2015)** demonstrated that nanocomposite compound (nanocurcumin+ surfactant+ ZnONPs+ vitamin C) causes no significant decrease in NO levels which were elevated in experimentally tumor bearing female mice due to cancer after 2 and 4 weeks.

In the present study, light microscope examination of sections of the lung of Zinc Oxide Nanoparticles intoxicated group showed collapsed alveoli, destruction of interalveolar septa with alveolar dilatation, thick interalveolar septa, and heavy infiltration of inflammatory cells, inflammatory exudate and extravasation of RBCs in the interstitium.

The results of the present study coincided with those of **Song et al. (2010)**, **Shokouhian et al. (2013)** & **Valdiglesias et al. (2013)** who investigated the toxic effects of ZnONPs in rats by oral administration resulting in enhancement of phagocytosis by macrophages causing

lymphocytic perivasculitis, pibronchiolitis and interstitial pneumonia, interstitial fibrosis, contraction and atelectasis of parenchymal lung tissue, patterns of chronic necrotic bronchiolitis and excretion of exudates in rat lung. It is also documented that ZnO nanoparticles could elevate the level of lactate dehydrogenase (LDH) and induce inflammation and apoptosis. Also, Thickening of alveolar septal wall thickening, vascular congestion, inflammatory infiltration and focal loss of epithelial integrity was reported following exposed to small doses (1mg/m³) of ZnONPs by inhalation for short period (5 days) **Wesselkamper et al. (2001)**

Moreover, **Jacobsen et al. (2015)** reported that exposure of mice to zinc oxide nanoparticles (0.3 mg/kg) cause strong pulmonary toxicity with cell death, epithelial damage, desquamation and injury to the alveolar barrier allowing fluids to enter the pulmonary space.

Yousef and El-Rajhi, (2013) attributed lung injury caused by ZnONPs to oxidative stress and free radical damage.

Also, **Zidan, (2011)** explained thickening of interalveolar septa induced by ZnONPs by the increased interstitial collagen fiber deposition and marked cellular infiltration with lymphocytes, neutrophils, eosinophils and macrophages. Moreover, vascular congestion and cellular infiltration of the lung tissue could be caused by changes of the vascular integrity of the lung vessels causing disruption of the endothelial barrier and increased capillary permeability.

Furthermore, **Nel et al. (2009)** demonstrated that uptake of ZnONPs in

acidic lining of lung cells accelerates dissolution of these particles leading to lysosomal damage, mitochondrial disturbance, production of ROS and cytokines.

Those results are in consistence with the findings of the study as GSH; biomarker of oxidative stress was decreased significantly in ZnONPs treated rats. Also, NO; an inflammatory mediator was increased significantly as a compensatory mechanism occurs with pulmonary inflammation and oxidative damage.

The light microscope examination of sections of the lung of ZnONPs+AA group revealed partial improvement in histopathological changes. These results are in agreement with, **Nemenqani, (2015)** who reported that rats treated with Vit c along with ZnONPs show well preserved lung tissue with partial improvement in lung histo pathology. Also, vitamin C administration (30mg/kg) with ZnONPs was found to decrease congestion and inflammatory infiltration in the tissues. **Nemanqani et al. (2015)**

Moreover, **Fukui et al. (2015)** mentioned that AA is a radical-scavenging antioxidant has the ability to inhibit the induction of pulmonary oxidative stress, injury and inflammation observed in the rat lung 1 day after intratracheal instillation of ZnONPs.

The comet assay is a widely used assay in ultimate research for DNA damage and repair, genotoxicity testing of novel chemicals and pharmaceuticals and environmental bio monitoring. However, comet assay has been working for toxicity assessment of highly reactive nanoparticles; and several studies had used it to

investigate the potential toxicity of manufactured nanoparticles by assessing DNA strand breaks or oxidative DNA lesions (**Karlsson, 2010 & Karlsson et al., 2015**).

In this study the effect of ZnONPs was demonstrated at molecular level by Comet assay to investigate the ability of ZnONPs to generate DNA damage and cause long term side effects in offsprings. Our results showed that administration of ZnONPs at dose 400mg/kg/ day for 4 weeks causes DNA strand breaks and disrepair of damaged DNA strands evidenced by increase in unit tail moment.

These results are in accordance with **Sharma et al. (2009); Yang et al. (2009); Osman et al. (2010); Sharma et al. (2011) ; Guan et al. (2012) and Dubey et al. (2015)** who reported that different concentrations of ZnONPs are cytotoxic and genotoxic to different organs and cell lines in different organisms. This genotoxic effect reported to be dose dependent **Muserrat et al. (2009)** and even after short term exposure **Gerloff et al. (2009)**.

These results could be explained by **Singh et al. (2009) & Yang et al. (2009)** who stated that increased ROS induced by nanoparticles in lysosomes can cause DNA point mutations or induce single-or double-strand breaks. Due to their small size; nanoparticles accumulate around the nucleus and few of them may diffuse through nuclear pores from where protein transport take place and this augment DNA damage caused by ROS (**Simko et al., 2011**).

Also, **Hausladen and Stamler (1999) & Murphy, (1999)** concluded that unregulated production of NO can lead to damage of cellular proteins, DNA damage, cell injury and death and

this may be a cause of DNA damage induced by ZnONPs.

In addition, the presence of free zinc ions may be the cause of ROS-driven cytotoxicity and genotoxicity (Song et al., 2010).

However, other studies noted weak or no genotoxic effect for ZnONPs when internalized into bacteria Kumar et al. (2011) & Nam et al. (2012). But Pan et al. (2010) stated earlier that attributed weak mutagenic effect of ZnONPs in bacteria to reduced particle size (bare ZnONPs < 100 nm), low dose response and low uptake of NPs.

The results of this work showed no-significant difference in mean values of unit tail moment in ZnONPs + AA group when compared with ZnONPs intoxicated group, but there was highly significant increase when compared with negative control group.

These results are in agreement with the findings of Bhat et al. (2006) who reported that several in vitro experiments with cell lines indicate that vitamin C in the presence of transition metal ions acts as a prooxidant and increases the amount of damage to genetic material in human lymphocytes.

In addition, Wang et al. (2014) mentioned that co-incubation of gastric epithelial cell lines and neural stem cells with ZnONPs and vitamin C lead to significant increase in the average fluorescence intensity in cells measured by DNA fluorescent staining indicating nuclear and mitochondrial damage.

These results suggest that vitamin C has a minimal protective effect on ZnONPs induced DNA damage and these results could be explained by low dose of vitamin C, short duration of supplementation or the fact that increased DNA damage needs long

time with sufficient doses of vitamin C for the tissue to restore normal genetic and chromosomal appearance (Bhat et al., 2006).

On the contrary to our results, Blasiak and Kowalik, (2001); Blasiak et al. (2004); Robichova et al. (2004) and Arranz et al. (2007) evaluated the protective effect of vit C against several free radicals generating chemicals (N nitrosamine, acrylamide and ZnONPs) by numerous in vivo and in vitro studies. Vitamin C significantly decreased DNA damage proved by Comet analysis.

CONCLUSION

Oral administration of 1/20 LD₅₀ of ZnONPs led to decrease in serum GSH as an indicator of ZnONPs induced oxidative stress and increased levels of NO as a compensatory mechanism resulting from oxidative stress. Also, it causes DNA damage evidenced by Comet assay with histopathological changes in lung. Ascorbic acid administration along with ZnONPs led to attenuation of oxidative stress and pulmonary toxicity induced by ZnONPs, but there was non-significant improvement in DNA damage proved by Comet assay.

RECOMMENDATION

Zinc oxide nanoparticles should be used cautiously to gain the benefits of nanotechnology and avoid its possible drawbacks. Public education regarding the health hazards of ZnONPs products should be adapted. Control occupational exposure to ZnONPs by continuous monitoring of work environment level and keep it within the recommended exposure limits. Increasing the awareness of workers about the proper handling of ZnONPs materials with periodical clinical and laboratory examinations are needed.

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دور حمض الأسكوربيك فى التسمم الرئوى الناجم عن جسيمات أكسيد الزنك النانوية ر فى ذكور الجرذان البيضاء البالغة

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ان جسيمات أكسيد الزنك النانوية تستخدم على نطاق واسع فى المنتجات الاستهلاكية والصناعية، وخاصة فى مستحضرات التجميل، المضافات الغذائية والصناعات الضوئية والمطاطية. ولقد أجرى هذا البحث لدراسة دور حمض الاسكوربيك ضد التسمم الرئوى القصير المدى المزمع الناجم عن جسيمات اكسيد الزنك النانوية فى ذكور الجرذان البيضاء البالغة. وقد تم تقسيم 60 من ذكور الجرذان البيضاء البالغة الى خمس مجموعات و هى المجموعه الاولى (الضابطه السالبه و الموجبه) و المجموعه الثانيه(مجموعه حمض الاسكوربيك) وقد تكونت من 10 من ذكور الجرذان البيضاء البالغة وتم اعطاؤها حمض الاسكوربيك (20مجم/كجم) لكل جرذ مذاب فى 1 مل من الماء المقطر عن طريق الفم مرة واحدة يوميا لمدة 30 يوما و المجموعه الثالثه (مجموعه جسيمات أكسيد الزنك النانوية)وتكونت من 10 جرذان وتم اعطاؤها جسيمات اكسيد الزنك النانوية (400مجم/كجم) لكل جرذ مذاب فى 1 مل من الماء المقطر عن طريق الفم مرة واحدة يوميا لمدة 30 يوما و المجموعه الرابعه (مجموعه جسيمات اكسيد الزنك النانوية + حمض الاسكوربيك)وتكونت من 10 جرذان تم اعطئهم الجرعات السابقه من حمض الاسكوربيك و جسيمات اكسيد الزنك النانوية معا لمدة 30 يوما. وقد اظهرت النتائج ان جزيئات اكسيد الزنك النانويه تسببت فى انخفاض ملحوظ فى نسبة الجلوتاثيون المختزل مع زياده فى اكسيد النيتريك وتلف ملحوظ فى الحمض النووى لخلايا الرئه تم رصده بجهاز فحص الحمض النووى الكهربائى للخليه الواحدة مع تجمع للخلايا الالتهابيه فى المنطقه المحيطة بالقصيبيات الهوائية والاويعه الدمويه، احتقان فى الانسجه بالاضافه الى تليف ما بين خلايا الرئه عند فحصها بالميكروسكوب الضوئى بينما ادى إعطاء حمض الاسكوربيك مع جسيمات اكسيد الزنك النانوية الى تحسن ملحوظ فى هذه الآثار السامة ولكن حمض الاسكوربيك لم يكن كافيا لتحسين التسمم الجينى. وبالتالي فان جسيمات اكسيد الزنك النانويه لها تأثير سمي على الرئه ويتحسن جزئيا بتناول حمض الاسكوربيك حيث انه لم يحسن التلف الناتج بالحمض النووى .