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Lactoferrin Ameliorates the Lung Toxicity Induced by Zinc Oxide Nanoparticles in Adult Male Albino Rats

Hanan I. El-Kerdasy1, Haidy M. Fakher2 , Hend R. Mousa3

1,3Department of Anatomy and Embryology, 2Department of Forensic Medicine and Clinical Toxicology Benha Faculty of Medicine, Benha University, Egypt

Corresponding author: Hanan I. El-Kerdasy, **Telephone:** 01007618122,

E-mail address: mhanan909@yahoo.com**, ORCID:** 0000-0001-9172-0289

ABSTRACT

Background: Zinc oxide nanoparticles (ZnO-NPs) are one of the most commonly used oxide nanoparticles. However, lung toxicity has been reported dependent on concentration and time of exposure. Lactoferrin (LF) exhibits both antioxidant and anti-inflammatory properties. **Objective:** The study aimed to identify toxic influence of ZnO-NPs on lung tissue & the possible ameliorative effect of lactoferrin on lung toxicity in adult albino rats.

Materials and methods: Forty adult male albino rats were randomly separated into 4 groups: Group I represented the control group, group II was given LF (300 mg/kg/day) orally for 3 weeks, group III was intraperitoneally injected with ZnONP 1000 mg/kg once, and group IV received LF and ZnONP concomitantly in the same dose and manner as groups II & III respectively. The lung specimens were handled for different biochemical, histological and immunohistochemical examination. Morphometrical and statistical studies were also performed. **Results:** ZnONP group showed disorganization of lung architecture that was verified by biochemical and histological examination. There was significant increase of the mean area % occupied by collagen fibers and immunopositive reaction of caspase 3 and NF-κB in ZnONP group. Lactoferrin administration in group IV presented improvement of all the previous results & oxidative stress biomarkers.

Conclusion: ZnONP induced lung injury, while lactoferrin ameliorated the toxic effect of ZnONP.

Keywords: Lactoferrin, lung, ZnONP, caspase 3 and NF-κB.

INTRODUCTION

Nanoparticles are mostly distinct as elements at the size from 1to 100 nm. Zinc oxide nanoparticle (Zo.NPCs) is one of the most profuse metal oxides, it is cheap, harmless, and it is simple to prepare [(1)]**.** Nanoparticles have become widely used because they are used in a wide range of industries, such as electronics, biomedicine, medicines, and cosmetics. For now, their poisonous and harmful side effects remain increased [(2)]. As a result, human exposure to NPs and its detrimental effects became inevitable [(3)]. Zo-NPs are common metal nanoparticles with antibacterial qualities; they have been used to prolong the freshness of food and stop it from spoiling. It is used in preparation of sunscreens as they have a powerful capacity to block ultraviolet radiation [(4)]. Consequently, Zinc oxide nanoparticle is therefore commonly used commercially as a component in food additives & packaging, medication administration, cancer treatment and water remediation. Biosafety studies on the toxicity of nanoparticles have increased [(5)]. Many studies reported that oral administration of Zo NPs has toxic effect on lung, spleen, brain, testes and liver. The pathological mechanisms for their toxicity are oxidative stress, initiation of inflammation, deposition of fibrosis with damaging DNA [(6)]. Lactoferrin is an endogenous pleiotropic particle. It is a multifunctional iron-binding glycoprotein, it is recognized as lactotransferrin, it has antibacterial & antiviral properties, profuse in airway secretions, it is considered in the protection against airborne pathogens [(7)]. Adding, lactoferrin has immunomodulatory, antioxidant, antitumoral and anti-inflammatory properties. Lactoferrin is able to be used in treatment of many diseases, it is often reflected as beneficial agent and assistant to drug-based treatments of lung diseases [(8)]. Consequently, the goal of this work was to identify the poisonous influence of zinc oxide nanoparticles on lung tissue, and the possible ameliorative role of lactoferrin on lung injury in adult albino rats.

MATERIAL AND METHODS

Rats: A total of forty male adult albino rats, weighed between 180 and 200 g, were used in the present research. The animals were procured from the Faculty of Veterinary Medicine, Benha University, and maintained in stainless-steel cages under standard laboratory conditions at a temperature of 25°C with a 12-hour light/dark cycle. To ensure adaptation to the laboratory environment and diet, all rats were permitted a one-week acclimatization period before the commencement of the experiment. The experimental work was performed in the Department of Anatomy, Faculty of Medicine, Benha University, Egypt.

MEDICATIONS

Zinc oxide nanoparticles:

ZnONPs were obtained from Sigma-Aldrich Chemical Corporation, Egypt in the procedure of a flask of 100 g and then dissolved in distilled water before use. To define the size and shape of the nanoparticles, **Transmission Electron Microscopy (TEM)** was done. A droplet of the nanoparticle solution was put onto grids and permitted to dry through evaporation of water at room temperature. The TEM images were captured by a **JEOL JEM-1010 transmission electron microscope** operated at 80 kV at

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the **Regional Center for Myology and Biotechnology (RCMB), Al-Azhar University** (figure 1).

Lactoferrin (LF):
Pravotin bovine lactoferrin (100 mg/sachet) was obtained as a powder sachet from Hygint Pharmaceuticals Company. It is manufactured in Egypt (Green field 6 industrial zone, Alexandria. Each sachet powder was dissolved in 5 ml distilled water each ml contained 20 mg lactoferrin.

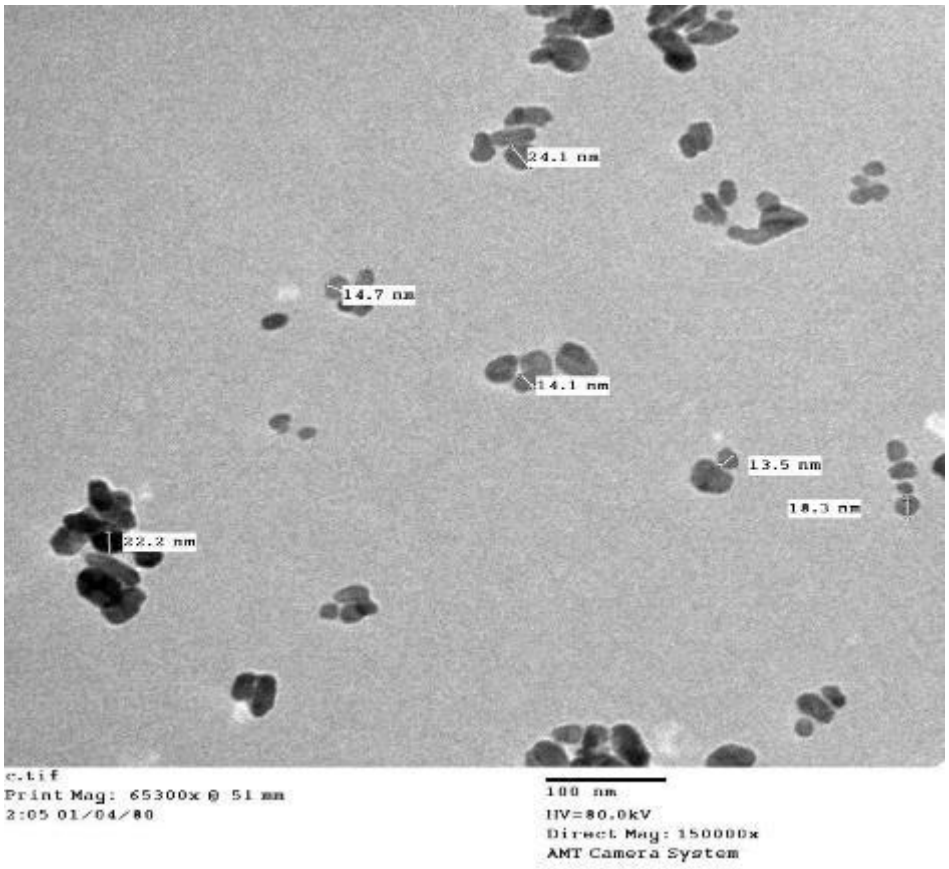

Fig (1): A transmission electron micrograph of ZNONPs suspension showing that the prepared zinc oxide nanoparticles were in the nanoscale range with a particle size less than 24.01 nm (TEM x15000).

Experimental groups

- **Group I (Control):** Contains ten rats that did not receive any medication, only taken a standard diet.
- **Group II (Lactoferrin group):** Contains ten rats that taken lactoferrin 300 mg/kg orally by gastric tube once every day for 3 weeks [(9)].
- **Group III (ZnONP group):** Contains ten rats that taken 1000 mg/kg zinc oxide nanoparticles single intraperitoneal IP injection (as a single toxic dose in the experiment) [(10)].
- **Group IV (ZnONP + Lactoferrin group):** contains ten rats that taken 1000 mg/kg/day of zinc oxide nanoparticles single IP injection & lactoferrin 300 mg/kg orally by gastric tube once daily for 3 weeks [(10)].

Finally, all animals were sacrificed by cervical displacement, then we opened thoracic cavity by abdomen-thoracic incision, lung were extracted & collected right lungs for biochemical analysis and left lungs for histopathological and immunohistochemical examination.

Tissue preparation for biochemical examination: Lung specimens were homogenized in RIPA buffer while maintained on ice. The resulting homogenates were centrifuged at 4°C for ten minutes, after which the supernatants were gathered for biochemical examination. The levels of superoxide dismutase (SOD), malondialdehyde (MDA), glutathione peroxidase 1 (GPX1), tumor necrosis factor-alpha (TNF-α), and interleukin-6 (IL-6) were determined conferring to instructions by commercially existing assay kits (CUSABIO, USA; Catalog Nos. CSB-E08555r, CSB-E11987r, and CSB-E04640r) [(11)].

Histological examination: Lung specimens were installed in 10% buffered formalin, handled & inserted in paraffin blocks. These were then divide up at 5µm thickness thereafter stained with Hematoxylin and Eosin (H & E) for a classical histology examination. Masson Trichrome stain to establish stromal modifications in collagen fibers assessment [(12)].

Immunohistochemical examination: Anti-caspase-3 monoclonal rabbit antibody (1:200, #ab184787, Abcam) for recognition of apoptotic cells and Nuclear Factor kappa B (NF-κB). Paraffin-embedded lung specimens were processed for the avidin– biotin–peroxidase technique was employed to detect anti-caspase-3 & expression of Nuclear Factor Kappa B (NF- κB).
The 4 µm-thick paraffin slices were deparaffinized in xylene, then rehydrated using arranged ethanol, then preserved for 30 minutes by 0.3% H_2O_2, then by using a microwave the sections heated in citrate buffer (pH 6) for 15 minutes, 10% goat serum was applied to the portions for 30 minutes. Following a PBS wash, the tissue slices were raised with primary antibodies-a rat monoclonal anti-caspase 3 & anti-NF-κB antibody-for a whole night at 4 °C. Then the slides were counterstained with sections stained with Mayer's hematoxylin, dehydrated, and fixed using DPX. Negative controls were organized by excluding the primary antibody during incubation. Positive immunoreactivity was identified microscopically by the presence of a characteristic brown coloration in the immunoreactive cells [(13)].

Morphometric analysis: The percentage of the main area engaged by Masson-Trichrome, anti-caspase 3 immunostained cells & NF-κB immuno-expression was quantified in ten haphazardly selected, non-overlapping microscopic fields per slide at 400× magnification for all lung specimens. By using an image analysis system (Leica Qwin 500 software, Image Computer System Ltd., Cambridge, England) at the Department of Anatomy and Embryology, Benha University.

Ethical approval: All procedures were obtained from the Scientific Research Ethics Committee of the Faculty of Medicine, Benha University (Approval No. RC 17-1-2026). Throughout the study, animal handling and care were conducted in accordance with the institutional guidelines of the Faculty of Medicine, Benha University, and followed the recommendations of the Guide for the Care and Use of Laboratory Animals issued by the US National Institutes of Health (National Research Council, 2011).

Statistical study

All data were examined by the IBM SPSS software package, version 20 (Armonk, NY, USA), and existed as the mean ± SD. The statistical analysis was achieved by ANOVA and Tukey's post hoc test for pairwise evaluations. A p-value of ≤ 0.05 indicated significant relations.

RESULTS

Biochemical analysis: ZONPs presented higher oxidative stress, proved by significant rise ($P < 0.05$) in malondialdehyde (MDA) levels in ZONPs group in comparison with control, lactoferrin and ZONPs + lactoferrin groups. And lowered the antioxidant enzymes activity as there were significant reduction in superoxide dismutase (SOD) and glutathione peroxidase 1 (GPX1) levels in ZONPs group compared to control, lactoferrin and ZONPs + lactoferrin groups. Correspondingly, after treatment with lactoferrin a significant reduction ($P<0.05$) was present in malondialdehyde (MDA) level in ZONPs + lactoferrin group in comparison with ZONPs group and significant rises ($P<0.05$) were present in SOD and GPX1 levels in comparison with ZnO-NPs group. Rats exposed to ZONPs exhibited elevation in the inflammatory mediators as there were significant rises ($P < 0.05$) in tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) levels in ZONPs group compared to control, lactoferrin & ZONPs + lactoferrin groups. After administration of lactoferrin, there was significant decline ($P<0.05$) in tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) levels in ZONPs + lactoferrin group compared to ZONPs group (Table 1).

Table (1): The biochemical parameters among all studied groups

	Group I	Group II	Group III	Group IV
MDA nmol/mg	0.8 ± 0.08	0.9 ± 0.04 [c & d]	6.99 ± 0.3 [a ,b& d]	1.98 ± 0.8 [a,b &c]
SOD U/mg	195.5 ± 3.8	196.5 ± 3.8 [c & d]	66.7± 3.5 [a ,b& d]	178.99 ± 5.7 [a,b &c]
GPx U/mg	238.2 ± 2.9	241.8 ± 3.4 [c & d]	93.7 ± 1. 8 [a ,b& d]	180.03 ± 6.4 [a,b &c]
TNFα pg/mg	50.4 ± 1.02	47.9 ± 0.8 [c]	447.3 ± 8.9 [a ,b& d]	117.2 ± 8.8 [c]
IL6 pg/mg	48.2 ± 0.99	47.3± 1.1 [c & d]	453.8 ± 3.3 [a ,b& d]	92.3 ± 3.6 [a,b &c]

Data stated as mean ± SD, ***:** significance ≤ 0.05 (Tukey's test) **a:** Significance vs Control, **b:** Significance vs group II, **c:** Significance vs group III, **d:** Significance vs group IV.

Histological study:

H & E stained lung sections from control & lactoferrin groups were similarly presented normal histological structures of the lung with normal alveoli that were surrounded by thin 1ry inter alveolar septa with 2ry septa were projected into alveolar sacs. Normal bronchi with simple columnar epithelium and normal blood vessel (Figure 2a & b). While ZNONPs group showed disturbed lung architecture with diffuse collapsed alveoli, thick interalveolar septa, areas of exudate were seen extravasated within the lung tissue, and severe inflammatory cells infiltration. The bronchi showed epithelial cell lining and cellular debris inside lumen of bronchiole. Congested thick wall blood vessel was seen (Figure 2c & d). ZNONPs + lactoferrin group showed that majority of the alveoli had recovered where their inflation with several alveoli appeared normal and some alveoli were collapsed. The1ry interalveolar septa were clearly thin in some places and others were mildly thickened. 2ry septa were projected into alveolar sacs. The bronchioles appeared normal and its epithelial lining was restored with minimal cell desquamation in the lumen. Some areas of exudate were seen extravasated within the lung tissue (Figure 2e). Masson's Trichrome (MT) stained lung sections from the control and lactoforrin groups had normal blue staining of collagen fibers distribution around bronchi (Figure 3 a & b).

In contrast, ZNONPs group showed lung sections with severe massive blue staining of collagen fibers distributed around bronchi and within the lung interstitium in alveolar wall (Figure 3c). ZNONPs + lactoferrin group section showed moderate blue staining of collagen fibers distribution around bronchi and blood vessels (Figure 3d).

Immunohistochemical study:

Caspase 3 immunohistochemical staining in lung tissue from the control and lactoferrin groups had negative caspase 3 immunoreactivity (Figure 4a & b). Conversely, ZONPs group showed strong diffuse cytoplasmic caspase 3 immunoreactivity in thick alveolar wall and bronchi (Figure 4c). Section from the ZONPs + lactoferrin group had moderate cytoplasmic caspase 3 immunoreactivity in alveolar wall (Figure 4d).

Regarding NF-kB immunohistochemical staining in lung tissue from the control and lactoforrin groups, only a few alveolar cells displayed a weak immunopositive brown reaction (Figure 5a & b). However, ZONPs group showed a strong positive brown cytoplasmic NF-κB immuno-expression in several alveolar cells and bronchi (Figure 5c). Section from the ZONPs + lactoferrin group had a moderate positive brown cytoplasmic immuno-expression in some alveolar cells (Figure 5d).

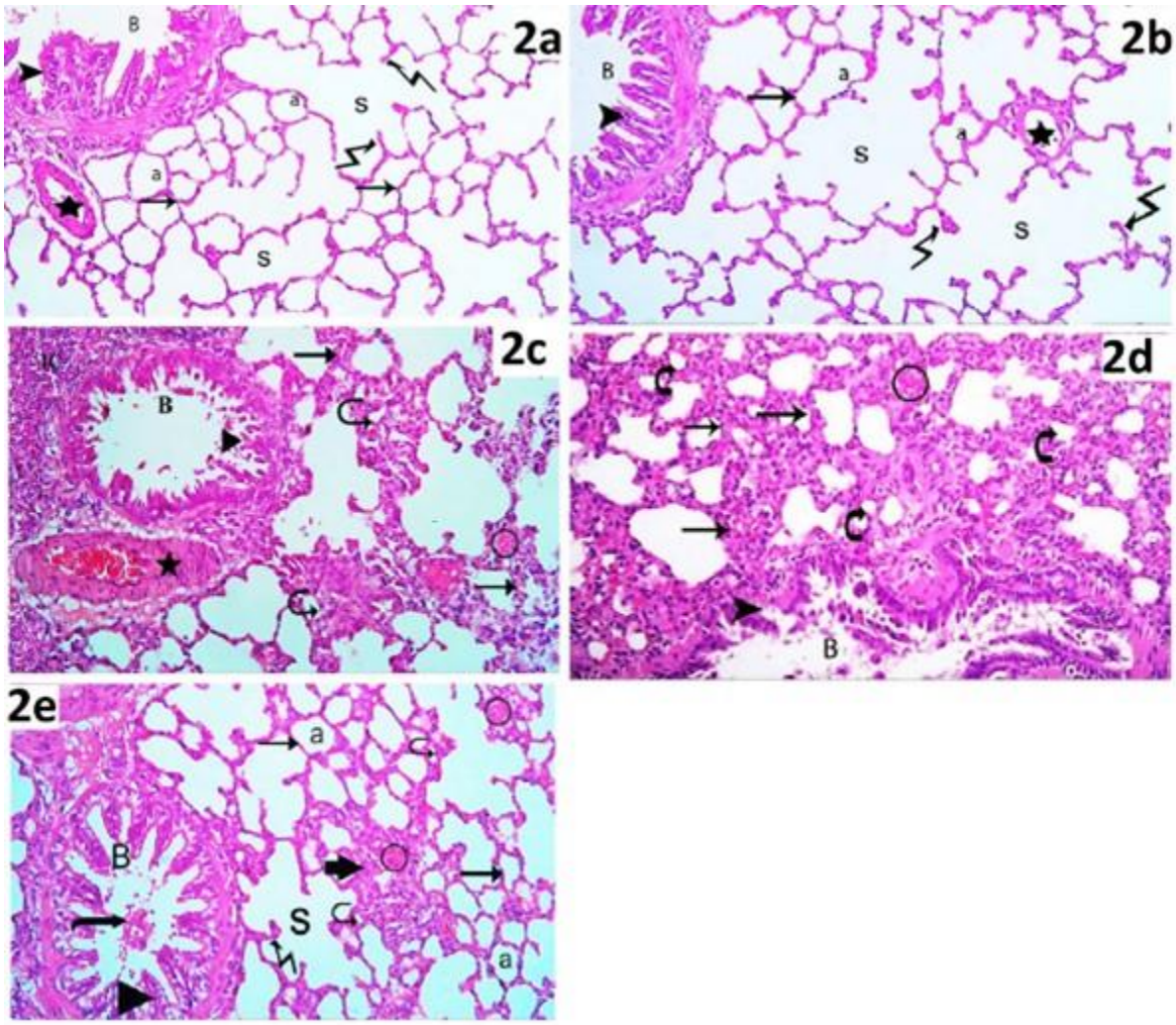

Figure (2): Photomicrographs of lung sections **(2a):** The control group showed normal lung histological architecture with alveoli (a) with thin1ry interalveolar septa (arrow), 2ry septa (zigzag arrow) introduced into alveolar sacs (s), normal bronchi (B) with simple columnar epithelium (arrow head), and blood vessel were seen (star). **(2b):** The lactoferrin group showed normal lung histological architecture with alveoli (a) are surrounded by thin1ry interalveolar septa (arrow), while 2ry septa (zigzag arrow) were projected into alveolar sacs (s), with normal bronchiole (B), with simple columnar epithelium (arrow head) and blood vessel were seen (star). **(2c):** The ZNONPs group showed disturbed lung architecture in the form of thick interalveolar septa (arrows) and collapsed alveoli (curved arrow). Some areas of exudate were seen extravasated within the lung tissue (circle). Intrabronchial cellular debris (arrow head) can be observed inside lumen of bronchioles (B). Sever inflammatory cells infiltration (IC) and congested thick wall blood vessels (star) were seen. **(2d):** The ZNONPs group showed abnormal organization of the lung tissue in the form of diffuse alveolar damage with numerous collapsed alveoli (curved arrow). Thick interalveolar septa (arrow), with areas of exudate were seen extravasated within the lung tissue (circle), with detached epithelial cell lining (arrow head) of the bronchioles (B). **(2e):** The ZNONPs + lactoferrin group showed that the majority of the alveoli had recovered inflation with some normal alveoli (a) and some collapsed alveoli (curved arrow), the1ry interalveolar septa were clearly thin (arrow) in some places and mild thickened (thick arrow) in others. 2ry septa (zigzag arrow) are projected into alveolar sacs (s). The bronchioles (B) were apparent normal and its epithelial lining is restored (arrow head) with minimal cell desquamation in the lumen (tailed arrow). Some areas of exudate are seen extravasated within the lung tissue (circle) (H & E ×200).

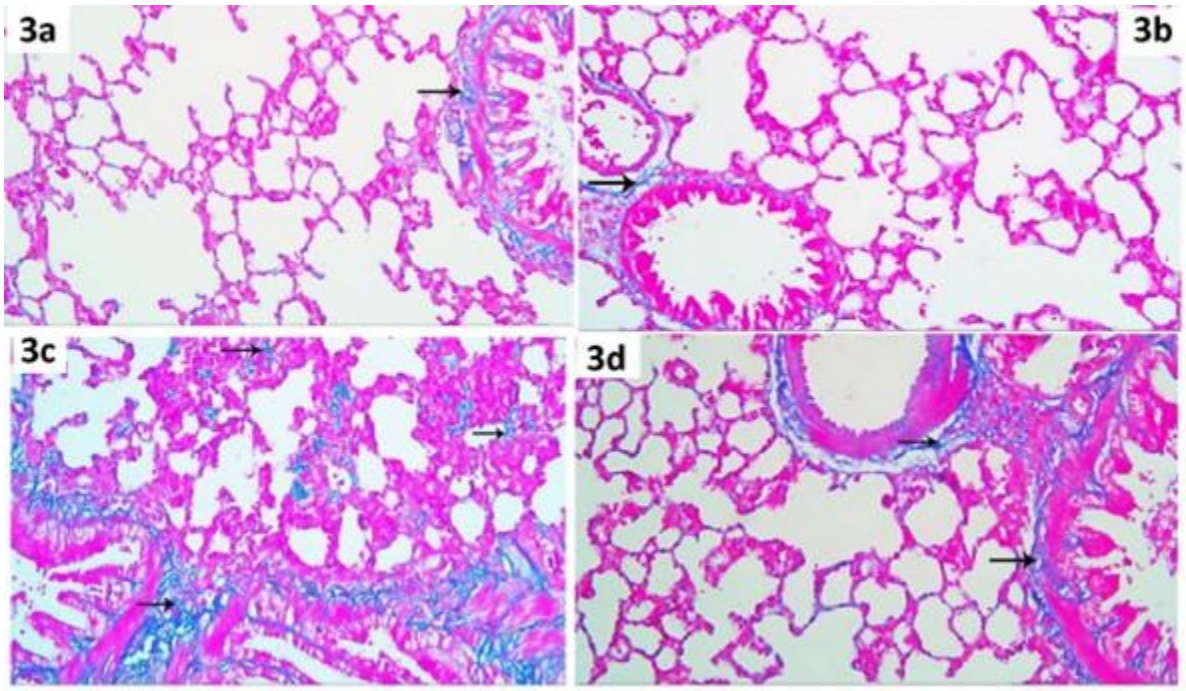

Figure (3): Photomicrographs of lung sections; **(3a):** The control group showed normal blue staining of collagen fibers distribution around bronchi (arrow). **(3b):** The lactoferrin group showed normal blue staining of collagen fiber distribution around bronchi and blood vessel (arrow). **(3c):** the ZONPs group showed sever massive blue staining of collagen fibers distribution around bronchi and within the lung interstitium in alveolar wall (arrow). **(3d):** from the ZONPs + lactoferrin group showed moderate blue staining of collagen fibers distribution around bronchi and blood vessels (arrows) (MT X 200).

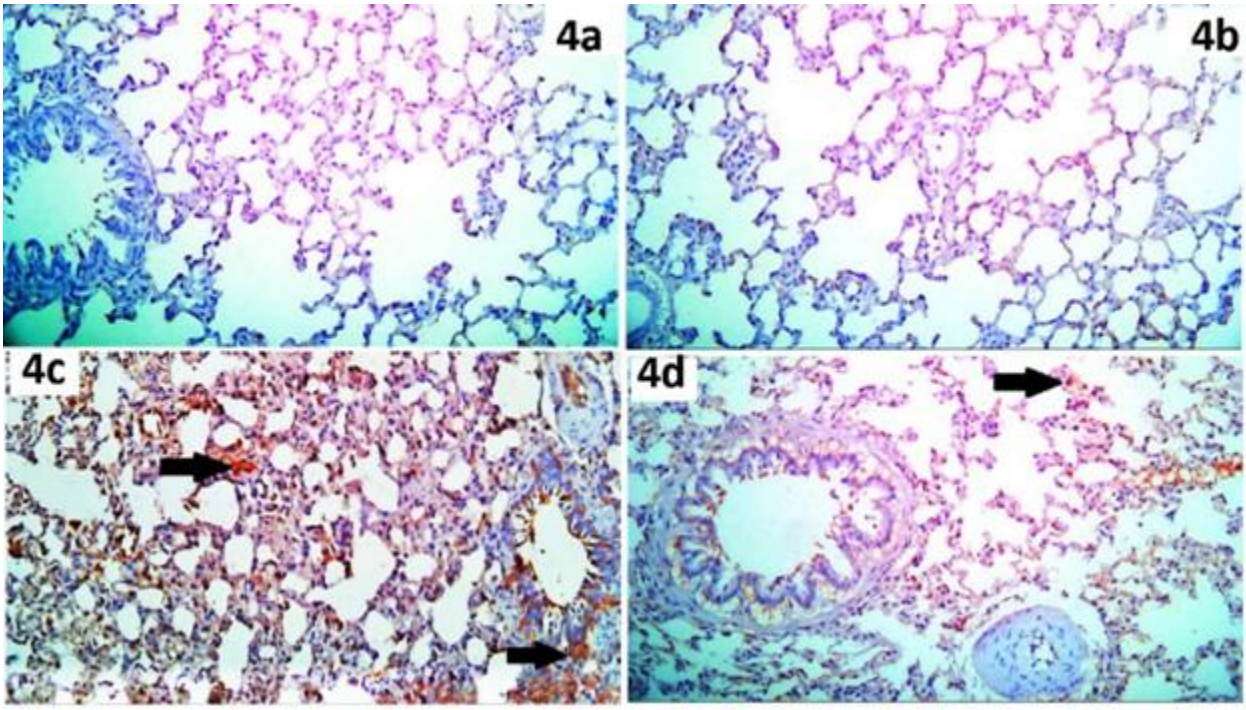

Figure (4): Photomicrographs of lung sections; **(4a):** The control group showed negative caspase 3 immunoreactivity in lung tissue. **(4b):** The lactoferrin group showed negative caspase 3 immunoreactivity in lung tissue. **(4c):** The ZONPs group showed strong diffuse cytoplasmic caspase 3 immunoreactivity in thick alveolar wall and bronchi (arrows). **(4d):** The ZONPs + lactoferrin group showed moderate cytoplasmic caspase 3 immunoreactivity in alveolar wall (arrow) (caspase 3 immunoreaction X 200).

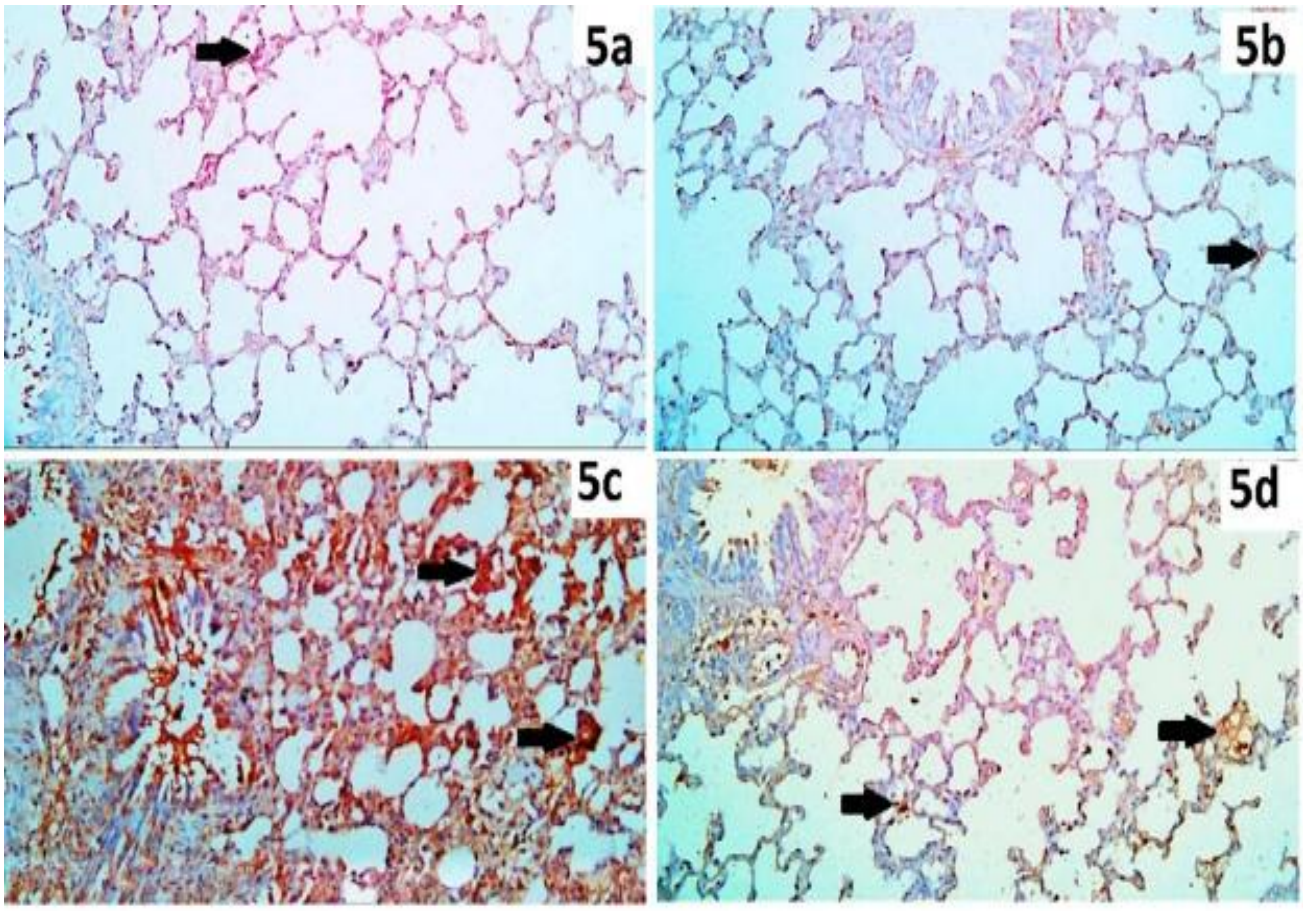

Figure (5): Photomicrographs of lung sections; **(5a):** The control group showed few alveolar cells that displayed a weak immunopositive brown reaction (arrow). **(5b):** The lactoferrin group showed few alveolar cells displayed a weak immunopositive brown reaction (arrow). **(5c):** The ZONPs group showed a strong positive brown cytoplasmic staining the NF-κB immuno- expression in several alveolar cells and bronchi (arrows). **(5d):** The ZONPs + lactoferrin group showed a moderate positive brown cytoplasmic staining the NF-κB immuno-expression in alveolar cells (NFKB immunoreaction X 200).

Morphometric study: Statistical evaluations of the main area % of collagen fiber distribution, caspase 3 immunoreactivity and NF-kB immunoexpression in all groups established a significant rise ($p \leq 0.05$) in the main area percentage of collagen fiber distribution in ZONPs group in comparison with control, lactoferrin and ZONPs + lactoferrin groups. There was a significant decline ($p \leq 0.05$) in ZONPs + lactoferrin group compared to ZONPs group. Caspase 3 immunoreactivity and NFKB immunoexpression showed significant rise ($p \leq 0.05$) in ZONPs group compared to control, lactoferrin and ZONPs + lactoferrin groups, while significantly declined ($p \leq 0.05$) in ZONPs + lactoferrin group compared to ZONPs group (Table 2).

Table (2): The averages main area % of collagen fibers, caspase 3 and NF-kB ± SD for all studied groups

Mean area % ± SD	Group I	Group II	Group III	Group IV
Masson area %	1.2 ± 0.3	1.2 ± 0.3 [c]	11.9 ± 1.9 [a, b&d]	2.09 ± 0.4 [c]
Caspase 3 area%	2.03 ± 0.8	2.05 ± 0.8 [c]	21.5±1. [a, b&d]	2.4± 0.6[c]
NF-kB area %	1.9 ± 0.96	1.8 ± 0.7 [c]	27.9 ± 3.9 [a, b&d]	2.5 ± 1.2 [c]

Data stated as mean±SD, ***:** significance ≤ 0.05 (Tukey's test) **a:** Significance vs Control, **b:** Significance vs group II, **c:** Significance vs group III, **d:** Significance vs group IV.

DISCUSSION

The lung is believed to be more vulnerable to oxidative damage than any other part of the body. Because of its constant exposure to air that may contain dangerous particles or reactive chemicals [14]. Exposure to ZnO-NPs causes lung oxidative stress and cell damage by oxidase enzyme activation, lipid peroxidation, elevated nitric oxide levels, and autophagy induction [15]. Numerous variables, including size, dosage, mode of administration, and length of exposure, have been found to affect ZnO-NPs toxicity [16]. Lactoferrin acts as a protecting, anti-inflammatory mediator contrary to lung injury, mainly by decreasing oxidative stress. It lessens destruction of acute lung toxicity [17].

In this study ZONPs existed elevation in oxidative stress, where there was a significant upsurge in MDA levels in ZONPs group in comparison with control, and lowering of the antioxidant enzymes activity appeared as significant decrease in SOD and GPX1 levels in ZONPs group compared to control and lactoferrin groups. This is in agreement with **Lai-Bao *et al.*** [18] who found that extreme reactive oxygen species were formed in the lung of rats inspired ZnO-NPs and diminution in SOD level and other antioxidant activity.

In this study, rats exposed to ZONPs displayed rise in the inflammatory mediators as there were significant rise in TNF-α and IL-6 levels in ZONPs group compared to control and lactoferrin groups. Consistent with our study some authors reported that inflammatory reaction is an essential feature of ZnO-NPs-enhanced acute lung damage, exposure to ZnO-NPs augmented the amounts of inflammatory cells as neutrophils, macrophages and lymphocytes [15]. Similar results were obtained by other authors who added that ZnO-NP-stimulate reactive oxygen species formation prompted the increase of damaged mitochondria leading to release interleukin -1 beta [18]. However, another study stated that after rats were inhaled ZnONP at a dose of 10 mg/ m^2 for one month produced a temporary elevation in neutrophil influx in pulmonary tissue [19].

On the other hand, administration of lactoforin in group IV improved the biochemical parameters as there was a significant decline in MDA levels in comparison with ZONPs group with significant increase in SOD and GPX1 levels compared to ZnO-NPs group. **LI *et al.*** [20] added that lactoferrin repressed the pulmonary inflammation induced by lipopolysaccharide via inhibition of the toll-like receptor 4–related pathway. Lactoferrin reduced the release of inflammatory mediators in lung tissue, where there was a significant decrease in TNF-α and IL-6 levels in ZONPs + lactoforin group compared to ZONPs group. Also, they revealed that lactoferrin actually decreased the releases of IL-1β and TNF-α from lung tissues in pulmonary inflammation.

In this work H & E stained sections from ZnONPs group presented abnormal disturbed lung architecture with diffuse collapsed alveoli, thick interalveolar septa, areas of exudate were seen extravasated within the lung tissue, and sever inflammatory cells infiltration. The bronchi had detached epithelial cell lining and cellular debris was observed inside the lumen of bronchioles, with congested blood vessels. Our findings are closely agreeing with the results of **Mohammed *et al.*** [21] who found widespread alveolar collapse, thickened interalveolar septa and collapsed alveoli, with signs of interstitial consolidation. Correspondingly, **Junge *et al.*** [22] stated that increased thickness of alveolar wall was due to neutrophilic infiltration in the alveolar wall. **Alsemeh *et al.*** [10] added that ZnONP revealed ultrastructural abnormalities, as the air-blood barrier was disturbed, type I pneumocytes and endothelial cell were irregular with swollen cytoplasm. The increasing oral doses of ZnONP induced significant injuries to the lung tissue with elevation TNF-α and IL-6 levels and these injuries may be irreversible [23]. Furthermore, **Zidan** [24] described the presence of areas of exudate within the lung tissue might be due to changes of the vascular integrity producing disturbance of the endothelial barrier and augmented capillary permeability, the exudate is frequently complemented by a major invasion of polymorphonuclear neutrophils and macrophage in the interstitial tissue and in the alveolar space.

In our study lactoferrin enhanced improvement in histopathological structures of lung tissue in group IV, which showed that the majority of the alveoli had recovered their inflation with several alveoli appeared normal and some alveoli were collapsed. The1ry interalveolar septa were clearly thin in some places and others were mildly thickened. 2ry septa were projected into alveolar sacs. The bronchioles appeared normal and its epithelial lining was restored with minimal cell desquamation in the lumen. Some areas of exudate were seen extravasated within the lung tissue. This is in agreement with **Marzouk *et al.*** [25] who stated that H & E sections from lactoferrin-treated group showed the lung tissue structures closely like to the control group. The interalveolar septa were thin, most of the alveoli were patent. Mild mononuclear cellular accumulation was noticed around some bronchioles. Most bronchioles have intact epithelium, but, some exfoliation of the lining epithelium was detected in a few bronchioles. Lactoferrin might be efficiently applied in the management and as a recovery supplement for COVID-19, due to its antiviral properties [26].

In the present study Masson trichrom stained lung sections from ZNONPs group have sever massive blue staining of collagen fibers distribution around bronchi & within lung interstitium in the alveolar wall and there was a significant elevation in main area % of collagen fiber

distribution in ZNONPs group in compared with control and lactoforrin groups. In the same line another study stated that lung sections from the ZnO-treated group presented an obvious increase in collagen distribution, there was dense fibers highly surrounding blood vessels, bronchioles and in the interstitial tissue [25]. In the same line **Osman *et al.*** [27] added that by electron microscopic study there were interstitial fibroblasts and collagen deposition in the interalveolar septa. Fibroblasts appeared as branched cells showing euchromatic nuclei that secret collagen. The thick interalveolar septa had extensive great areas of collagen deposition adding to congested blood capillaries.

In this work administration of lactoferrin in group IV, Masson Trichrome sections showed moderate collagen fibers distribution around bronchi and blood vessels, this improvement confirmed by a significant decline in main area % of collagen fibers in group IV compared to group III. This is in the same line with another study that believed that lactoferrin had possible antifibrotic properties on several tissues as the lung, liver, heart, skin and kidney. This procedure may be due to its ability for controlling the inflammation and the synthesis of the extracellular matrix [28]. Lactoferrin can be used as an assistant factor during treatment of cases of liver fibrosis [29]. In this study caspase 3 immunostained sections from ZONPs group showed strong diffuse cytoplasmic caspase 3 immunoreactivity in thick alveolar wall and bronchi. This was established by a significant elevation in main area % of caspase 3 immunoreactivity in group III compared to control and lactoferrin groups. This finding evidenced the role of ZONPS in elevation of apoptosis in lung tissue. Similarly, another author stated that ZONPS lung sections presented high caspase 3 cytoplasmic expression in several inflammatory cellular accumulations and few in bronchiolar & alveolar cells [27].

In this experiment sections from the ZONPs + lactoferrin group showed moderate cytoplasmic caspase 3 immunoreactivity in alveolar wall confirmed by significant decrease in main area % of caspase 3 immunoexpression in group IV compared to group III. This finding proved the effective role of lactoferrin in ameliorating the apoptotic influence of ZONPs, which is in promise with **Francis *et al.*** [30] who found that in normal and non-cancerous cells, lactoferrin often displays anti-apoptotic properties. In immune cells throughout inflammation lactoferrin inhibits primary apoptosis by delaying ceramide generation and caspase activation [30]. **Hou *et al.*** [31] stated that lactoferrin in concentration of 10 µg/ml has a maximum inhibition role in osteoblast apoptosis. Lactoferrin prevent osteoblast apoptosis by regulation of the expression of insulin-like growth factor I.

In this work ZONPs group showed a strong positive brown cytoplasmic NF-κB immuno- expression in several alveolar cells and bronchi indicating inflammation. This finding was proved by significant elevation in NF-κB immunoexpression in ZONPs group compared to control and lactoferrin groups. These results are in agreement with another study, which found a significant increase in NF-κB immuno-expression in lungs treated with ZnO-NP [10]. Also, this is consistent with **Fan *et al.*** [32] who established the role of NF-κB in mediating nanoparticle-persuaded inflammation.

Although, with administration of lactoferrin in group IV the lung sections presented moderate positive NF-κB immuno-expression in some alveolar cells confirmed by significant decrease in NF-κB immunoexpresion in ZONPs + lactoforin group compared to ZONPs group indicating the effective role of lactoferrin in reduction of inflammations induced by ZONPs. Similarly, another study stated that lactoforrin delays the NF-κB signal transduction pathway that is profoundly responsible for initiation of the inflammatory reactions to infection and any tissue damage. It also depressed the expression of pro-inflammatory cytokines and stimulates the discharge of anti-inflammatory cytokines [33]. Another authors added that Lactoferrin reduced the accompaniment activation subsequent to inflammation, and restricts the damage to inflamed organs. It depresses the frequency of respiratory tract infections by augmenting immune function and stopping extreme inflammatory overreaction in lung tissue. So lactoferrin is used in all-inclusive and nutritional lines to sustenance conditions obvious by chronic inflammation or autoimmune diseases [34].

CONCLUSION

ZnONP in a high dose prompted lung injury and lactoferrin ameliorated the toxic effect of ZnONP.

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