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Pulmonary Histological Alterations Induced by 20 nm Silver Nanoparticles

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Abstract. Silver nanoparticles (SNPs) are widely invested in nanomedicine and consuming products due to their unique antimicrobial properties. However, little is known about the toxicity of these particles on human health. The present investigation was carried out to investigate the histological alterations induced in the lung tissues by 20±5 nm SNPs. Male albino Wistar rats were exposed to SNPs at a daily dose of 2 mg/kg for 21 days. Lung biopsies from all rats under study were subjected to histopathological examinations. Exposure to 20±5 nm SNPs induced the following pulmonary alterations: thickened alveolar wall, macrophages invasion and inflammatory cells infiltration, lymphatic follicles enlargement, pulmonary edema, alveolar hypersensitivity and interstitial congestion. Occasional atelectasis and fibrocytes proliferation were also detected. The findings of the present work might indicate that SNPs potentially trigger oxidative stress and alterations in the pulmonary tissues that may affect the function of the lungs.

Introduction

Due to their unique antimicrobial, antifungal and antiviral properties, SNPs are being utilized in many consuming and industrial products. Silver NPs have been rapidly involved in different sectors of the medical field including prevention, diagnosis and treatment together with other application [1]. While the number of commercial and industrial products employing SNPs was 30 in 2006, it has grown to 1300 by the beginning of 2014 in various application in one way or another [2]. Moreover, silver NPs are used in the manufacturing of alginate fibers and wound dressing used in treating wounds, burns, skin ulcers and catheter related infections [3-4]. Silver NPs are also used in coating medical instruments and tools together with commercial brands of sunscreen, medical masks, gels, cosmetics, bone cement, tooth paste, deodorants, dental resin composite and shampoo [4]. In addition, these fine particles are invested in medical cloth, footwear, athletic shirts and textile manufacturing to limit odor due to sweating [5-6]. Also, silver NPs have unique optical, electrical and thermal properties and are being used in biological and chemical sensors and in imaging of neural tissues [7].

Silver nanoparticles accumulate mainly in the vital organs with the mitochondria as a primary target [7,8-13]. The nano size of SNPs together with their high surface area to volume ratio enable them to enter the tissue components as biological molecules do [14]. Asharani et al [15] concluded that SNPs could induce DNA damage, increase chromosomal aberrations, and reduce ATP production. Other toxicological studies have demonstrated size-dependent genotoxic and cytotoxic consequences [16-18]. Toxicological investigations concluded that alterations induced by SNPs might be also related to the charge and functional groups on the surface of these particles and due to the release of silver ions that bind or interact with the tissue and cell components [15,19-22]. *In vitro* toxicological studies showed that SNPs could produce reactive oxidative species (ROS) and induce cytotoxicity with the possibility of mitochondrial damage and cytoskeleton disruption [23].

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According to Singh and Ramarao [24], SNPs can enter the cytoplasm and induce toxicity causing mitochondrial damage, apoptosis and cell death.

Exposure to silver NPs is becoming part of human life due to the wide use of these particles in medicine and industry, making these particles persisted in our environment with possible potential risk on human health and ecosystems. This wide application together with the novel properties of SNPs are fraught with concerns from environmental and occupational exposure. Accordingly, full attention is needed to be given towards safety and toxicological issues of SNPs prior considering their benefits in implementation with a need to address knowledge gaps in the field of nanotoxicity.

Lung tissues receive high blood flow and have high exposure to SNPs with long circulating residue [25]. Silver NPs were reported to cause significant oxidative stress and cytotoxicity that could reveal a high risk potential on the vital organs [26-29]. Some studies reported toxic effects of SNPs in the pulmonary tissue with relation to the size and time of exposure [30]. Moreover, previous reports indicated that 20 nm SNPs are more toxic than the larger ones due to their ability to cross cell barriers more easily [26-28,31]. Limited studies have been carried out on the pulmonary histological alterations induced by SNPs exposure. With this objective, the present work was carried out to investigate the histopathological alterations induced in the lung tissues by 20±5 nm SNPs.

Materials and Methods

Silver nanoparticles. Spherical 20±5 nm SNPs obtained from the Department of Nanotechnology of Laser Zentrum- Hannover, Germany, were used in the present study. The particles were dissolved in deionized water containing 0.1 mM sodium citrate for stabilization to prevent particles from aggregation. These particles were fabricated by picosecond-pulsed laser ablation in liquids (PLAL) method. The following tools were used: Yb-YAG commercial high-power slab laser (Edgewave HD40I, GmbH), picosecond-pulsed laser system (TruMicro 5250, Trumpf GmbH), providing 7 ps laser pulses at a wavelength of 1030 nm (beam diameter: 6 mm, beam profile: Gaussian) together with variable pulse energy and repetition rate (maximum pulse energy: 250 µJ, maximum repetition rate: 200 kHz). Repetition rate was varied by an integer divisor without changing pulse energy, allowing highly reproducible results. The ablation process was carried out as follows: a silver foil (8 x 8 x 0.1 mm; purity: 99.99%), (Goodfellow GmbH) was fixed within a self-constructed ablation chamber made of Teflon, filled with 35 ml of deionized water containing 0.1 mM sodium citrate, resulting in a liquid column of 10 mm. The laser beam was coupled into a galvanometric scanner laser (Hurry SCAN II-14, Scanlab AG), and focused through telecentric lens (Sill Optics) with a focal distance of 56 nm to allow deposition of laser pulses at controlled interpulse-distance on the silver target. Liquid stirring was applied by using a teflon rotor for continuous liquid agitation and quick removal of ablated nanoparticles from the process zone. Determination of laser spot size on the target for flounce calculation was performed according to Farkas and Geretovszky [32]. Ligand-free SNPs were generated by applying 250 µJ pulse energy, repetition rate of 33 kHz and laser fluency of 12.7 Jcm^{-2}. Target position was selected with respect to the focus position in air and to yield the highest ablation rates in deionized distilled water. The optical extinction spectra of the fabricated particles were measured by using UV-Vis spectroscopy (Shimadzu 1650), while the nanoparticles micrographs were obtained by the use of a scanning electron microscope (Quanta 400 FEG).

Animals and conditions. Twenty healthy male Wistar albino rats from King Saud University colony of the same age (10-12 weeks old) weighing 210-230 gm were used in the present study. The animals were housed at 24 ± 1°C, on 12 h dark/light cycle, randomly assigned and separately caged to one test groups and a control one (10 rat each).

Experimental Protocol. Following a period of stabilization (7 days), the control animals received single ip injection of 400 µl of deionized water containing 0.1 mM sodium citrate while the members of the test group received a daily ip injection of 400 µl of 20±5 nm SNPs (2 mg/Kg) for 21 days.

All animals were handled and all experiments were conducted in accordance with the protocols approved by King Saud University Animal Care Ethical Committee while the experimental procedures were carried out in accordance with international guidelines for care and use of laboratory animals.

Histological processing. All members of all groups were euthanized by cervical dislocation after 21 days of treatment. Fresh lung biopsy from both lungs of each rat of all groups were cut rapidly, fixed in neutral buffered formalin, dehydrated with ascending grades of ethanol (70, 80, 90, 95 and 100%), cleared in xylene, impregnated then embedded and blocked out in paraffin wax. The tissue processing was carried out by using automatic tissue processor (Thermo Shandon Company, Citadel 2000). Paraffin sections (4-5 µm) of the control and GNPs treated rats were prepared by using rotary microtome (American Optical Spencer 820), and stained according to Jarrar and Taib [33] with conventional histological techniques.

Microscopic examination. Histological sections of all rats under study were examined by using photomicroscope (Leica DM2500) equipped with digital camera (Leica LAS EZ IC80 HD).

Results

Silver nanoparticles characterization. As represented in Figure 1(a-d), the used SNPs demonstrated spherical morphology with size variation range of 10-40 nm where the maximum particle count was at 20±5 nm size. In addition, the UV-visible spectrum of the particles presented single symmetric absorption band with maximum peak of absorbance around ~ 400 nm that correspond the normalized absorption spectrum of SNPs. Furthermore, the citrate molecules in the colloid were stacked to nanoparticles surface preventing them from aggregation.

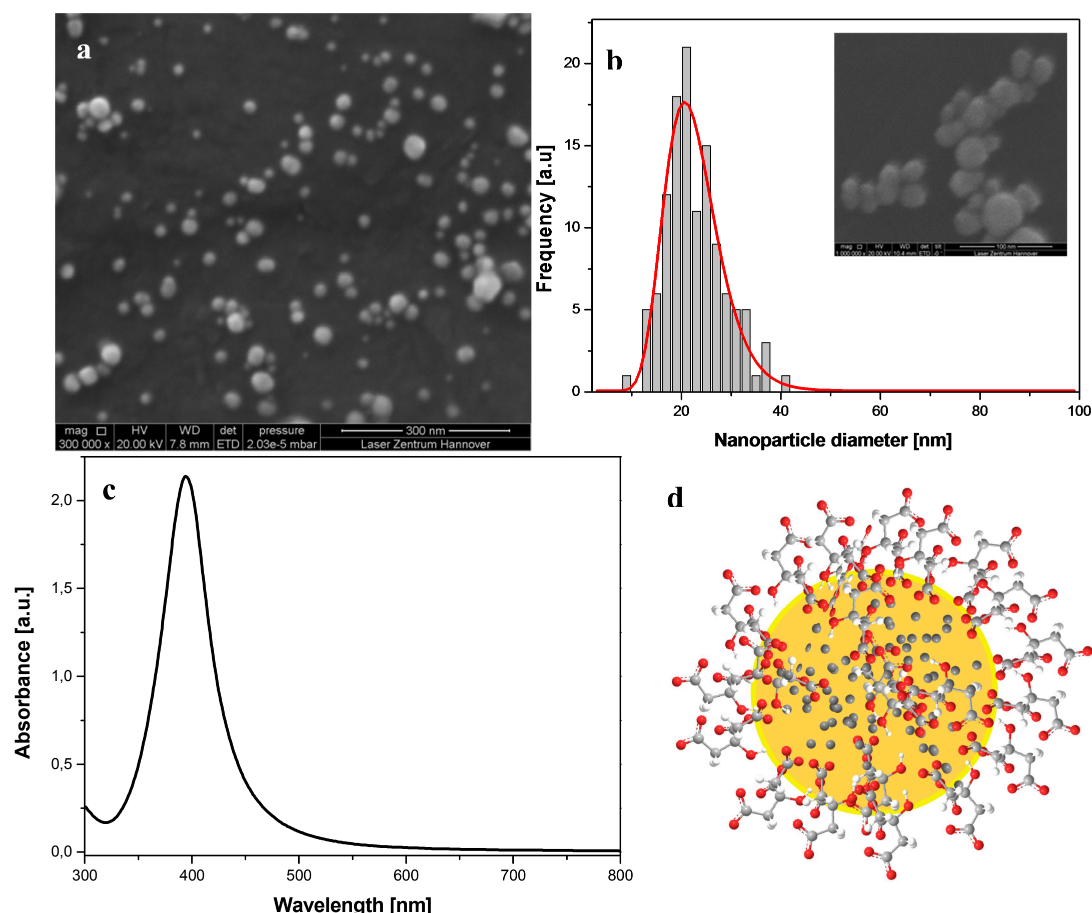


Figure 1(a-d). Physical characterization of the used SNPs demonstrating:
(a) Scanning electron micrograph (b) Histogram showing the particle size distribution of the particles (c) A plot UV-Visible spectrum of the particles
(d) Sketch of the administered SNPs with the citrate molecules on the surface

Lung of the control rats. Microscopic examination of the control rat lungs revealed normal alveolar architecture. The thin-walled alveoli consisted of simple epithelium surrounded by blood capillaries with normal distribution of pulmonary parenchyma vessels (Figure 2). The interalveolar septa of this group of rats were free from any abnormalities.

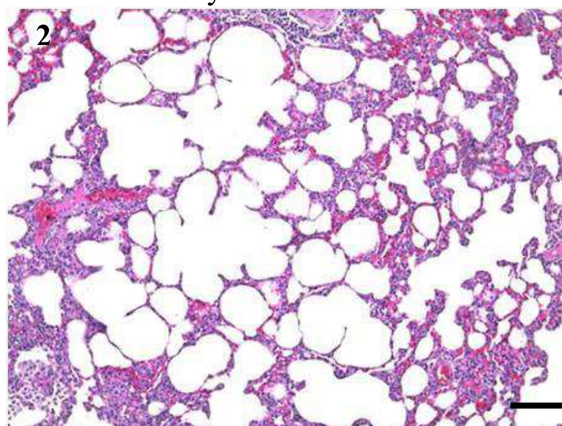


Figure 2. Light micrograph of section in the lung of control rat demonstrating normal lung tissue. H&E, Scale bar = 60 µm

Lung of rats treated with SNPs

Microscopic examination of lung tissues of rats exposed to 20±3 nm SNPs showed the following histopathological alterations:

Thickened alveolar walls. In comparison with control rats, the width of the tissue intervening between two adjacent pulmonary alveoli was increased. In addition, interalveolar septum thickening characterized by cellular exudates was observed with moderate congestion in the lungs of SNPs treated rats (Figure 3a-b).

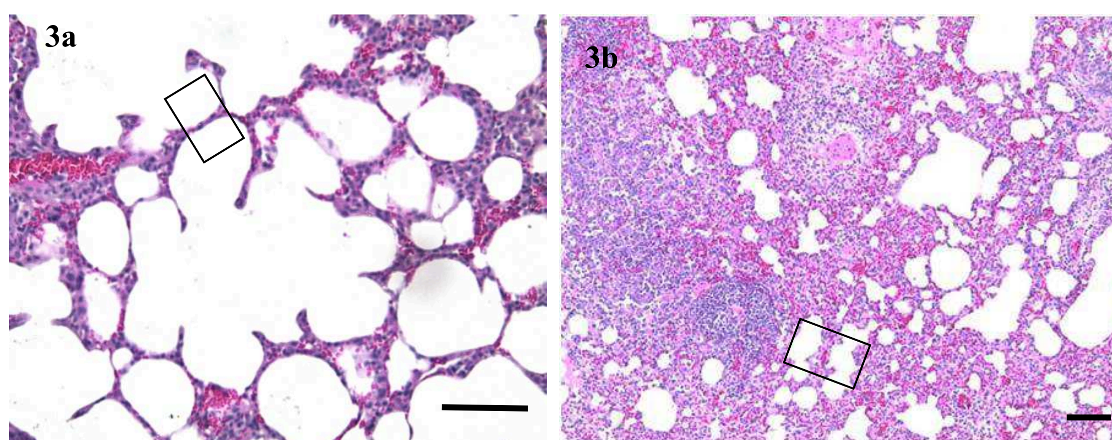


Figure 3(a-b). Light micrographs of sections in the lungs of**:** (a) Control rat demonstrating normal alveolar septa thickening (rectangle). H&E stain. Scale bar = **30** µm
(b) SNPs-treated rat demonstrating thickened alveolar septa (rectangle). H&E stain. Scale bar = 60 µm

Macrophages and inflammatory cells infiltration. Dense aggregates and diffused interstitial and peribronchial mononuclear inflammatory cell infiltration mainly lymphocytes was seen in the lungs of all members exposed to SNPs (Figures 4a-b). Plasma cells and eosinophils were also seen. Foamy interstitial alveolar macrophages were predominant together with other types of inflammatory cells in the ineralveolar interstitial tissue (Figure 4c).

Pulmonary lymphatic follicles. Silver NPs subjected rats demonstrated lymphoproliferative lungs. Enlarged lymphatic follicles were seen in the alveolar parenchyma in the lungs of these rats where some of the lymphoid follicles showed germinal center (Figures 5a-b).

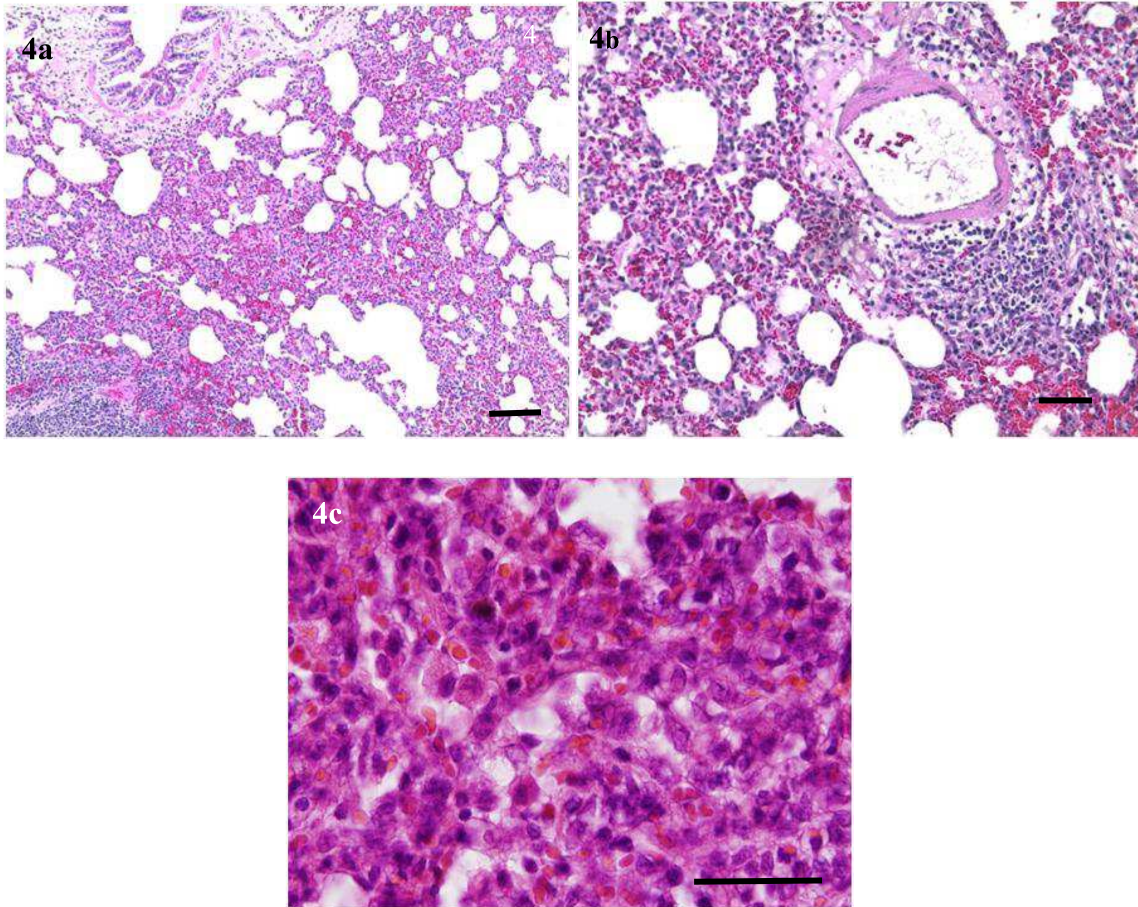


Figure 4(a-b). Light micrographs of section in the lung of SNPs-treated ratsdemonstrating:
(a) Interstitial mononuclear inflammatory cell infiltration. H&E stain.
Scale bar = 60 µm.
(b) Peribronchial inflammatory cell infiltration. H&E stain.
Scale bar = 60 µm.
(c) Alveolar septum heavy macrophages invasion. H&E stain.
Scale bar = 30 µm.

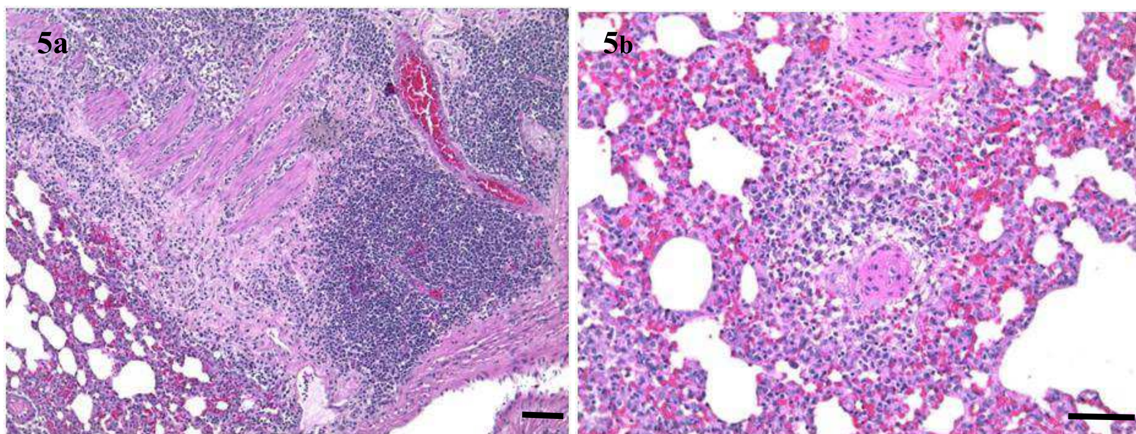
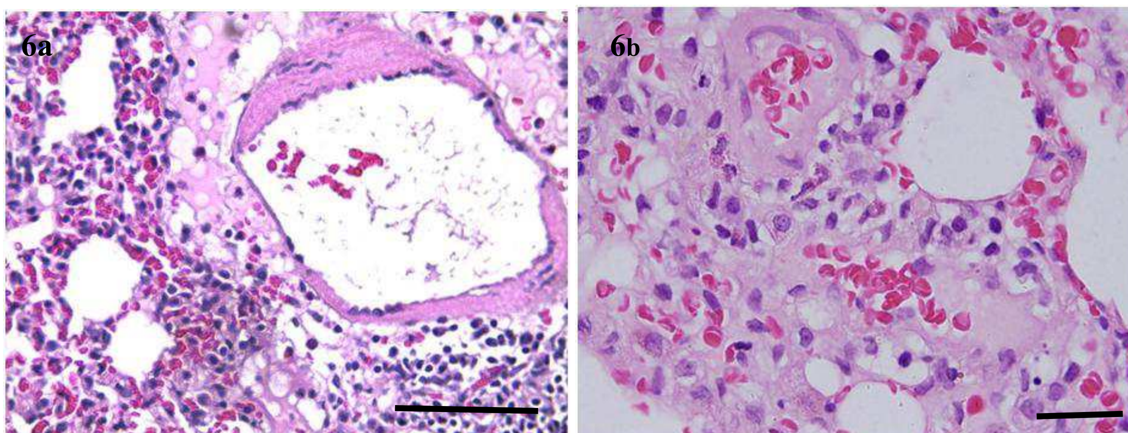


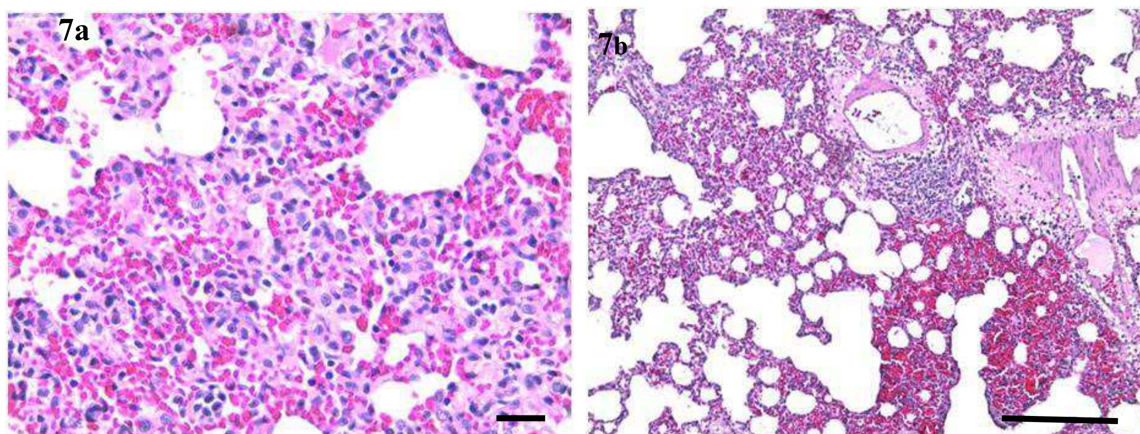
Figure 5(a-b). Light micrographs of sections in the lung of SNPs-treated ratsdemonstrating:
(a) Multiple pulmonary follicles. H&E stain. Scale bar = 60 µm.
(b) Pulmonary follicle with germinal center. H&E stain. Scale bar = 60 µm.

Pulmonary edema. Pulmonary interstitial eosinophilic edema in the lung tissue of all SNPs treated rats was observed (Figure 6a). Peribroncular pulmonary edema was also detected in the lung tissue of rats exposed to SNPs (Figure 6b).



Figures 6(a-b). Light micrographs of sections in the lung of SNPs-treated rats demonstrating:
(a) Interstitial edema (star). H&E stain. Scale bar = 60 µm.
(b) Peribronchial edema. H&E stain. Scale bar = 60 µm.

Interalveolar capillaries dilatation and congestion. Pulmonary congestion with dilated interalveolar septal capillaries and leakage of blood cells were seen (Figures 7a-b).



Figures 7(a-b). Light micrographs of section in the lung of GNPs-treated rats demonstrating:
(a) dilated interalveolar septal capillaries. H&E stain Bar= 100 µm.
(b) interstitial congestion. H&E stain. Scale bar = 60 µm.

Atelectasis

Focal narrowing and deflation of some alveolar sacs in the lung tissue of some SNPs treated rats were detected (Figure 8).

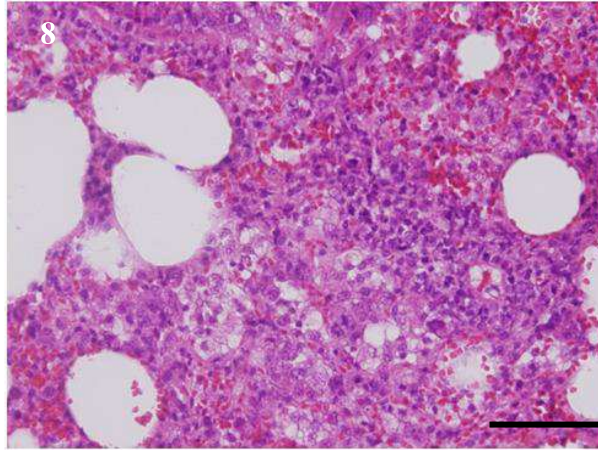
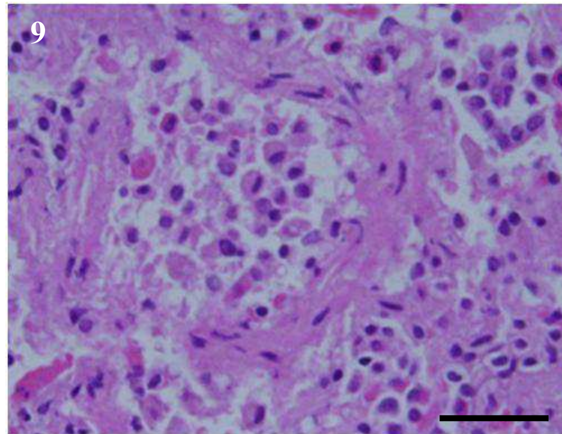


Figure (8). Light micrograph of section in the lung of GNPs-treated rat demonstrating atelectasis. H&E stain. Scale bar = 60 µm.

Alveolar hypersensitivity. Considerable number of eosinophils and plasma cells were seen in the thickened flamed interstitium and pulmonary blood vessels (Figure 9).



Figures (9). Light micrographs of section in the lung of GNPs-treated rat demonstrating considerable number of eosinophils and plasma cells in thickened flamed alveolar septum. H&E stain. Scale bar = 60 µm.

Fibrocytes proliferation. Spindle shaped fibrocytes were demonstrated in the alveolar septa of some rats exposed to SNPs (Figure 10). This proliferation was seen in the damaged inflamed thickened walls and accompanied by blood vessel thickening.

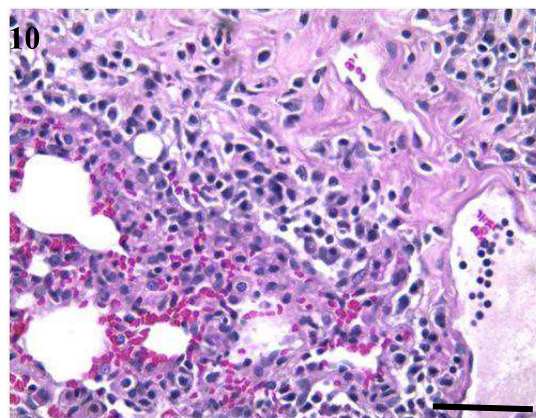


Figure (10). Light micrographs of section in the lung of SNPs-treated rat demonstrating fibrocytes proliferation together with emphysema and inflammatory cells infiltration. H&E stain. Scale bar = 60 µm.

Discussion

Silver NPs are potent constituents of pharmaceutical, medical and industrial products due to their unique optical, electrical and thermal properties. These particles have large functional surface area and can have the same dimensions of the biological molecules with the possibility of being adsorbed on the surface of these molecules in the tissues and cells [6]. Several reports indicated that SNPs release silver ions that may play key role in their toxicity by interacting with cellular organelles stimulating oxidative damage affecting cellular metabolism and membranes integrity via lipid peroxidation and protein denaturation [17, 34].

The findings of the present work indicate that daily intraperitoneal administration of SNPs to rats for 21 days at the concentration of 2 mg/km body weight, demonstrated variable pulmonary histological alterations in comparison with the control rats. The current results illustrated that SNPs could induce interalveolar septa thickening in the lungs o treated rats. This alteration might be resulted from the hypercellularity of the airspace walls induced by these particles toxicity. This finding is consistent with the previous reports where thickened epithelial alveolar walls were induced by inhalation of 18 nm SNPs [35].

The present investigation demonstrated inflammatory cells infiltration and macrophages pulmonary invasion due to SNPs exposure. Some previous studies reported inflammatory cells infiltration induced in the pulmonary tissues by SNPs exposure with transient changes in the pulmonary functions [20-21,35-38]. The predominance of macrophages due to SNPs exposure might indicate a defense mechanism to alterations in the local pulmonary environment and inflammatory phagocytes of cellular debris resulted from SNPs toxicity. Arai *et al* [39] suggested that SNPs could be transported to lysosomes and gradually dissolved in the macrophages. In addition, macrophages stimulate lymphocytes and other immune cells to respond to foreign substances and to participate in regeneration function [40]. This is in consistence with the results of the present study where SNPs-treated rats showed lymphoproliferative lungs. Moreover, the induced alveolar hypersensitivity by SNPs might indicate allergic alveolitis. Some previous studies indicated that SNPs could induce both neutrophilic and eosinophilic response and increased the chance of an asthmatic response [15].

The present work indicated that SNPs can induce moderate pulmonary edema. Lung tissue edema is an air spaces obstruction complication related to lung inflammation and pulmonary tissue fluid flooding. This finding might indicate that SNPs toxicity could induce hydrostatic forces on the alveolar capillary by increasing their permeability. The eosinophilic nature of the induced edema by SNPs subjection may indicate protein content. On the other hand, the appearance of extravasated erythrocytes in the alveolar sacs of SNPs-treated rats may indicate compression due to edema and/or thickening of the alveolar walls.

Moreover, the findings of the present work indicated atelectasis and fibrocytes proliferation in the pulmonary tissues of rats exposed to SNPS. Atelectasis might be resulted from partial blockage of alveoli in the affected area of the pulmonary tissue with interstitial exudates accumulation due to SNPs toxicity. This alteration may indicate respiratory complication that could prevent normal oxygen absorption to healthy tissues resulted from reducing elasticity of the pulmonary tissues [41].

The seen histopathological alterations in the pulmonary tissues might be related to the smaller nature of SNPs together with their high surface area to mass ratio that enable them to penetrate the tissue components easily [14]. Silver NPs toxicity might be associated to the charge and functional groups on their surface and due to the release of silver ions that are neutralized by binding or interacting with tissue components [19-22]. Some reports indicated that SNPs most probably induce oxidative stress by generating reactive nitrogen species (RNS) together with ROS [42-44]. Some *in vitro* toxicological studies indicated that SNPs could initiate production of ROS that could damage plasma membrane and cell organelles stimulating oxidative damage disruption [23]. Both ROS and RNS can cause DNA fragmentation, lipid peroxidation and protein dysfunction together with tissues injury [45].

The histological alterations observed in the present work may also indicate that exposure to SNPs could induce lung injury due to the interaction between SNPs with some biological components of the lung tissues. Nanoparticles less than 100 nm in diameter can enter cells while those less than 40 nm can enter the nucleus [46-47]. Some reports demonstrated size-dependent genotoxic and cytotoxic consequences such as DNA damage, chromosomal aberrations, cell cycle disturbance and metabolic activity reduction associated with SNPs exposure [19-21]. The used SNPs in the present study (size 20 nm) can enter the tissues and interact with the macromolecules of the pulmonary tissues specially proteins with possible adsorption of these molecules at the surface of the particles forming what is known as the nanoparticle-protein corona [48]. This together with probable heat alteration due to the plasmonic effect of the particles might result in structural changes of the adsorbed proteins in specific and the biological system as a whole.

One can conclude from the findings of the present work that SNPs exposure can cause oxidative stress resulting from disturbance in the pro-oxidant/antioxidant of the pulmonary tissues leading to tissue damage and affecting the function of the lung. In addition, the results of present study may raise the concerns about the potential risk on human health that might be related with numerous applications of silver nanoparticles. More work is needed to elucidate the potential risks of SNPs on the vital organs and their pathogenesis. The present study may suggest finding out whether coating or capping of SNPs can reduce their toxicity without affecting the activity of these nanomaterials.

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