Effect of Simvastatin on Hypervitaminosis D_3-Induced Changes in Lung and Trachea in Adult Male Albino Rats. Histological and Immunohistochemical Study

Mohamed A. Abdou Hegazy and Ghada G. Hamam

Histology Department, Faculty of medicine, Ain Shams University. Cairo, Egypt
ghada.hamam@yahoo.com

Abstract: Introduction: Vitamin D intoxication usually occurs as a result of inappropriate use or unnecessary prescription of vitamin D preparations. It can lead to life-threatening hypercalcemia. Statins are drugs widely administered to lower cholesterol & triglycerides serum levels. **Aim of the work:** to examine the effect of high dose vitamin D_3 on the structure of lung and trachea of adult male albino rats and to examine the effect of simvastatin on these changes. **Materials and methods:** twenty six adult male albino rats were used in this experiment. They were divided into three groups; control group, group II received vitamin D_3 by subcutaneous injection for three days. Group III: received simvastatin orally for ten days (four days before administration of vitamin D_3, during administration of vitamin D_3 as in group II (three days) and continued for further three days). At the end of the experiment, lungs and trachea were collected and processed for histological and immunohistochemical techniques. **Results:** administration of high dose vitamin D_3 resulted in deposition of calcium, extravasations of blood and mononuclear cellular infiltration in lung interstitium with significant increase in CD68 positive macrophages. Thickening of inter-alveolar septa with obliteration of some air spaces and dilatation of other alveoli were observed. Hypertrophy of bronchial epithelium and hyperplasia of goblet cells were also noticed. Tracheal epithelium showed intraepithelial lymphocytes. Lamina propria and submucosa showed cellular infiltration, extravasations of blood and hypertrophied submucosal glands. Hyaline cartilage showed deposition of calcium and fused empty chondrocytes lacuna. Pretreatment with simvastatin led to improvement of histological structure. **Conclusions:** administration of high dose vitamin D_3 produced metastatic calcification, mononuclear cellular infiltration, extravasations of blood and hyperplasia of mucous secreting cells in both trachea and lung with thickening of interalveolar septa. Pretreatment with simvastatin, ameliorated these changes.

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Key wards: hypervitaminosis D, statin, lung, trachea, osteopontin, CD68, rats

1. Introduction:

Vitamin D intoxication is a well-known cause of hypercalcaemia [1]. Overtreatment with vitamin D can have considerable consequences and is known to be toxic to humans, rats and other animals. In humans, manifestations of vitamin D toxicity include hypercalcemia, hypercalciuria, nausea, anorexia, lethargy, mental disturbances and ectopic soft tissue calcification [2]. High doses of vitamin D increase the supersaturation of calcium phosphate salts in the blood by increasing the absorption of calcium and phosphorus from the intestinal tract [3]. This promotes the uptake of calcium into the intracellular space, which leads to calcium overload and induces expression of genes for calcification [4]. Hypercalcemia is also a common finding that is associated with several pathological conditions. Causes of hypercalcemia include increase parathyroid hormone with subsequent bone resorption, destruction of bone in metastatic bone tumors or immobilization, vitamin D intoxication and renal failure which cases retention of phosphate, with subsequent secondary hyperparathyroidism [5].

Hypercalcemia usually leads to metastatic calcification. Metastatic calcification usually develops when plasma calcium and phosphorus concentration increase to the limit enough for calcium phosphate formation and precipitation [6,7]. Calcification can be either dystrophic or metastatic, with the former referring to calcium deposition in damaged tissue and the latter referring to calcium deposition in healthy tissue secondary to disturbance in calcium metabolism [5,8]. Many tissues, as heart, pancreas, lung, kidney, stomach, muscles, articular cartilage, intestine and blood vessels, are susceptible to ectopic calcification [9,10].

Metastatic pulmonary calcification is a subdiagnosed metabolic lung disease that is commonly associated with end-stage renal disease. It is seen at autopsy in 60–80% of haemodialysis patients. It is rarely recognized during life, but can progress to respiratory failure that is usually reflected in the survival of the patient [6,11,12]. Patients often complain of exertional dyspnea and bilateral, asymmetrical, interstitial infiltrations on chest X-ray [13]. Some patients develop severe respiratory

symptoms as restrictive lung diseases, chronic respiratory insufficiency and hypoxaemia or in some cases, lethal acute respiratory distress [6,8]. Pulmonary calcification can adversely affect both pulmonary and right ventricular function [14].

Prophylaxis of pulmonary calcifications is important as regression of established calcifications rarely occurs [9,12,13]. Statins, 3-hydroxy-3-methyl-gluratyl coenzyme A (HMG-CoA) reductase inhibitors, are effective in reducing vascular calcification in cardiovascular diseases [9]. It is hypothesized that statins could also reduce calcifications in the lung and trachea in case of hypervitaminosis D_3.

The aim of the present work was to study the effect of hypervitaminosis D_3 on the structure of lung and trachea of adult male albino rats, and to test the possible protective effect of simvastatin on these changes.

2. Materials and methods:

1-Animals and diet

Twenty six adult male albino rats with average weight 200 gm were purchased and raised in the Medical Research Center in Ain Shams University. Rats were housed in plastic cages with mesh wire covers under standard experimental conditions. They were given fresh tap water and standard food. All animal procedures were carried out according to the guidelines of animal care and the ethical committee of the faculty of Medicine, Ain Shams University**.**

2-Drugs and doses

Vitamin D_3 was given to rats by subcutaneous injection in a dose of 500000 IU/kg/day for three days [3]. It was obtained in the form of Devarol-S ampoules (Memphis pharmaceutical and chemical industries El-Amirya, Cairo, Egypt) containing 200000 IU cholecalciferol per 2ml ampoule.

Simvastatin was obtained in the form of Zocor tablets (manufactured by Merk Sharp and Dohma (MSD) UK). One tablet (10 mg) was suspended in five ml normal saline and was administered orally to rats by gastric tube in a dose of 10mg/kg/day for ten days [15].

3-Animal grouping

After one week acclimatization period, animals were randomly divided into three groups:

Group I (Control group): it consisted of ten rats that were further subdivided into two subgroups five animals each.

Subgroup Ia: received subcutaneous injection of one ml saline daily for three days, then they were sacrificed after further three days.

Subgroup Ib received one ml saline daily by gastric tube for ten days. On day five they received subcutaneous injection of one ml saline daily for three days. Then they were sacrificed after further three days.

Group II: (Vitamin D_3 group): consisted of eight rats that received subcutaneous injection of vitamin D_3 daily for three days. They were left without treatment for further three days then they were scarified.

Group III: (Simvastatin group): consisted of eight rats that received simvastatin daily by gastric tube for ten days starting four days before administration of vitamin D_3 as in group II. Simvasattin was continued during administration of vitamin D_3 (three days) and for further three days after vitamin D_3, and then rats were sacrificed.

4-Histological study

At the end of the experiment, all rats were sacrificed under ether inhalation anesthesia. Thoracotomy was done by lifting the sternum and cutting the ribs on both sides at their attachment to the costal cartilage. Intratracheal instillation of 0.5 ml 10% formalin was done till the lungs fill the chest cavity [16]. The whole trachea and the apical part of both lungs were taken and fixed immediately in 10% formol saline solution and were then processed to obtain paraffin sections of five µm thickness. Sections were subjected to H&E stain [17]. Von Kossa stain was also done to detect calcifications [18]. Areas with calcification appeared brown- black in color

Immunohistochemical stain

Sections of the lung were stained with anti-CD68 antibody (FLEX Ready-to-use, clone KP-1, Dako Omnis) for detection of alveolar macrophages. Positive reaction appeared as brown cytoplasmic reaction. Negative control was done by omitting the step of primary antibody, and positive control was done by using liver section.

5-Morphometric and statistical study

An image analyzer Leica Q win V.3 program installed on a computer in the Histology Department, Faculty of Medicine, Ain Shams University, was used. The computer was connected to a Leica DM2500 microscope (Wetzlar, Germany). Five different non overlapping fields from five different sections of different rats were examined in each group for measuring the mean area percentage of CD68 expression in the lung.

All measurements were taken at high-power fields of magnification (×20). All data were collected, revised, and subjected to statistical analysis using one-way analysis of variance performed using SPSS.21 program (IBM Inc., Chicago, Illinois, USA). P values less than 0.05 were considered significan

3. Results:

Histological results:

Lung:

Examination of ***H&E*** stained sections of the control group showed the lung formed of alveoli, alveolar sacs, respiratory bronchioles and bronchi. The wall of bronchi was seen formed of three layers, mucosa that was formed of simple columnar epithelium and thin layer of lamina propria, a layer of smooth muscle cells and adventitia. Alveoli were seen lined by pneumocytes and interalveolar septa were seen with thick part and thin part (Figure: 1). In vitamin D_3 group, inflammatory cells were seen inside the lumen of blood vessels (Figure: 2). Mononuclear cellular infiltration (Figures: 2,3) and extravasations of blood (Figure: 3) were seen in lung interstitium with narrowing of some alveoli and compensatory dilatation of other alveoli (Figures:2,3). Dilatation of bronchi were seen with hypertrophy of their epithelium and hyperplasia of goblet cells (Figures: 3,4). Inflammatory cells and mucous layer were seen on the surface of bronchial epithelium (Figure:4). Bronchus associated lymphatic nodules were frequently seen hypertrophied (Figure: 3). Thickened interalveolar septa were seen containing blood vessels with irregular thickening of their wall. Inflammatory cells were frequently seen adherent to these thickened wall (Figure:5). In simvastatin pretreated group, minimal amounts of cellular infiltration and extravasations of blood were seen in the interstitium. Most alveoli were seen patent (Figure: 6).

Examination of lung sections stained with ***Von Kossa*** stain, showed no calcium deposition in the control group (Figure: 7a), while in vitamin D_3 group brown calcium deposits were seen in the alveolar septa (Figure: 7b,7c). In simvastatin treated group, fine calcium deposits were seen in alveolar septa (Figure: 7d).

Immunohistochemical stain of lung sections using ***anti-CD68*** antibodies showed few positive cells in the control group (Figure: 8a). In vitamin D_3 group, aggregation of CD68 positive macrophages was seen in the alveolar septa (Figure: 8b). In simvastatin treated group, few positive cells were seen (Figure: 8c).

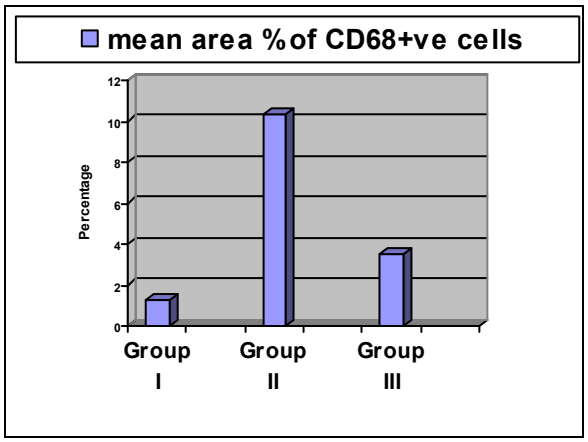
Trachea:

Examination of ***H&E*** stained sections of the trachea of the control group showed the wall formed of respiratory epithelium (pseudostratified columnar ciliated epithelium with goblet cells), lamina propria, submucosa, fibrocartiagenous layer and adventitia. The hyaline cartilage of the tracheal rings was seen covered with fibrous perichondium. Flattened chondrocytes, rounded chondrocytes, cell nests and basophilic matrix were also seen (Figure: 9). In vitamin D_3 group, irregular festooned appearance of the respiratory epithelium with intra epithelial lymphocytes were seen (Figure: 10). Cellular infiltration and extravasations of blood were frequently noticed in the lamina propria and the submucosa (Figures: 10,11). Loss of demarcation between the epithelium and lamina propria (Figure: 11) and hypertrophy of mucus glands in the submucosa (Figure: 12) were also noticed. Chondrocyte lacunae were seen irregularly fused and empty (Figure: 11). In simvastatin treated group, minimal amounts of cellular infiltration was noticed in the submucosa and chondrocytes were seen inside their lacuna (Figure: 13).

Examination of tracheal sections stained with ***Von Kossa*** stain showed no calcium deposition in the control group (Figure: 14a), extensive calcification was noticed in the tracheal rings of vitamin D_3 group (Figure: 14b), while in simvastatin group, no calcium deposition were seen in the hyaline cartilage (Figure: 14c).

Morphometric and statistical results:

In the current study, a significant (P<0.05) increase in the mean area percentage of CD68 positive reaction was noticed in the lung of vitamin D_3 group (group II) (10.1 ± 1.5) compared to control group (1.3± 0.4). Simvastatin pretreated group showed a significant decrease (3.5 ± 1.1) compared to vitamin D_3 group (Histogram 1).



Histogram 1: showing the mean area percentages of CD68 positive cells in different groups

4. Discussion

The common usage of large doses of vitamin D and Ca- containing phosphate drugs contribute to calcifications in soft tissues that leads to life-threatening complications [10].

It was reported that internal alkaline compartment predispose to metastatic calcification [5,8]. Blood pH in the lungs is more alkaline than in other organs particularly in the lung apices, where there is a higher ventilation-perfusion ratio which leads to greater PaO_2 and lower $PaCO_2$, and consequently, a higher pH [8]. This was the cause of

choosing lung apices to be examined in the current study.

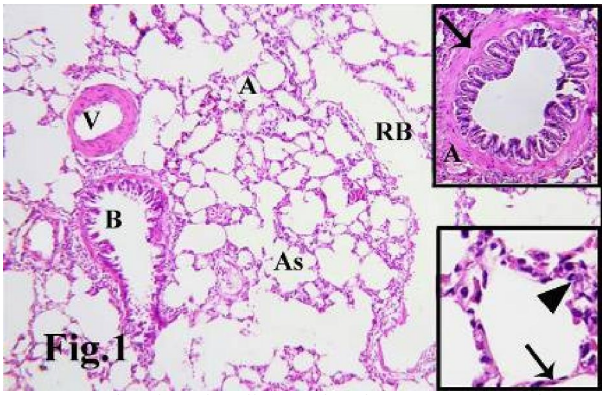


Figure 1: showing alveoli (A), alveolar sacs (As), respiratory bronchiole (RB), bronchiole (B) and blood vessels (V). The upper inset shows bronchus with simple columnar epithelium, muscle layer (↑) and adventitia (A). The lower inset shows thick part (▲) and thin part (↑) of interalveolar septa.
(Group I) H&E X100; Upper insetX100; Lower inset X640

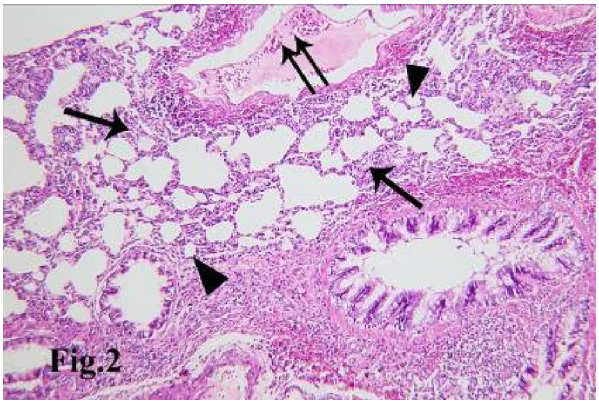


Figure 2: showing mononuclear cellular infiltration (↑) in lung interstitium with narrowing of some alveolar spaces (▲). Inflammatory cells are also seen inside the lumen of blood vessel (↑↑).
(Group II) H&E X100

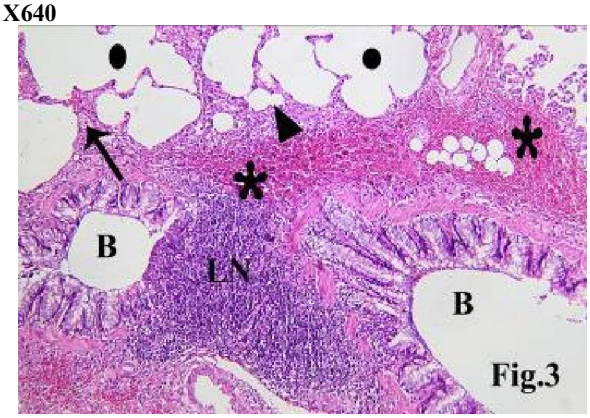


Figure 3: showing bronchi (B) with hypertrophy of their epithelium and enlargement of bronchus associated lymphatic nodule (LN). Mononuclear cellular infiltration (↑), and extravasations of blood (*) are also seen in the interstitium. Narrowing of some alveoli (▲) and dilatation of other alveoli (●) are noticed.
(Group II) H&E X100

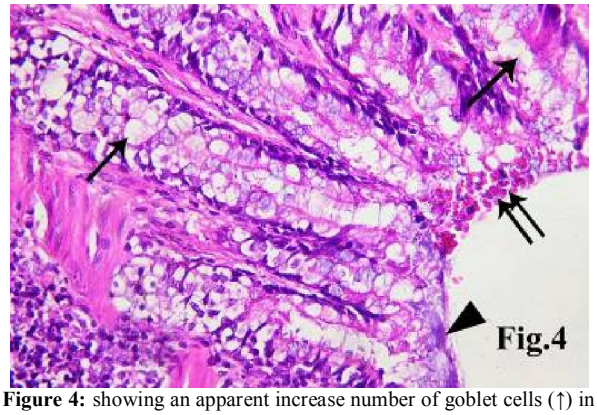


Figure 4: showing an apparent increase number of goblet cells (↑) in the lining epithelium of bronchus. Inflammatory cells (↑↑) and mucous layer (▲) are seen on the surface of the bronchial epithelium.
(Group II) H&E X640

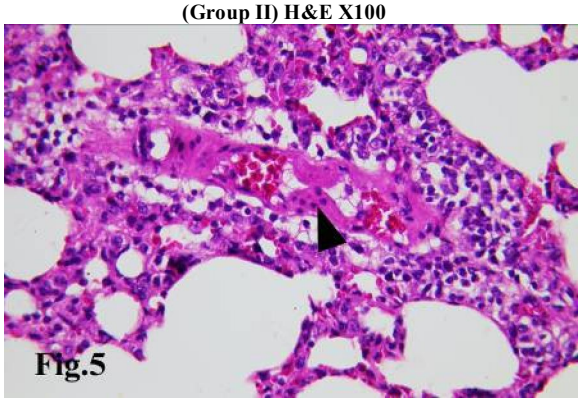


Figure 5: showing thick interalveolar septa containing blood vessel with thick wall. Adherence of inflammatory cells (▲) is also seen in the wall of blood vessel. **(Group II) H&E X640**

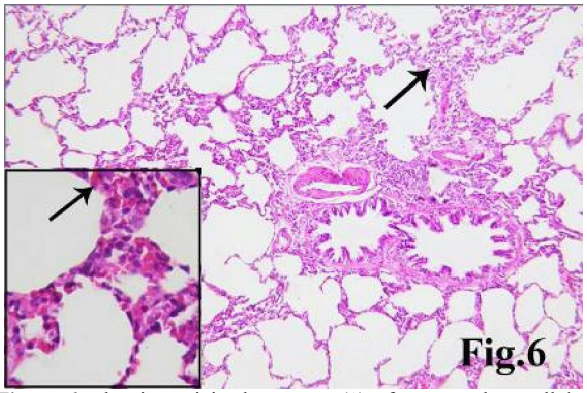


Figure 6: showing minimal amounts (↑) of mononuclear cellular infiltration and extravasations of blood (inset) in the interstitium. Most alveoli are seen patent. **(Group III) H&E X100, insetX640**

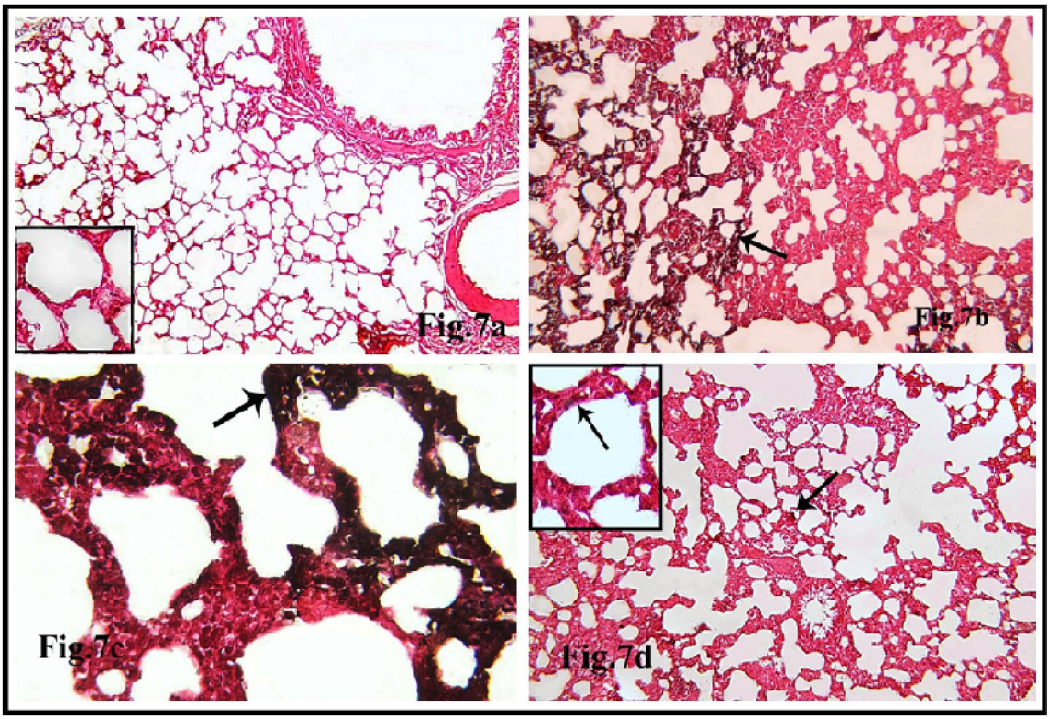


Figure 7: showing no brown calcium deposits in the alveolar septa (↑) of the control group (Figure: 7a). Brown calcium deposits are seen in the thickened alveolar septa (↑) (Figure: 7b, 7c) in vitamin D3 treated group. Fine calcium deposits are seen in simvastatin treated group (Figure: 7d).
Von kossa 7a, 7b, 7d X100, Insets and 7c X640

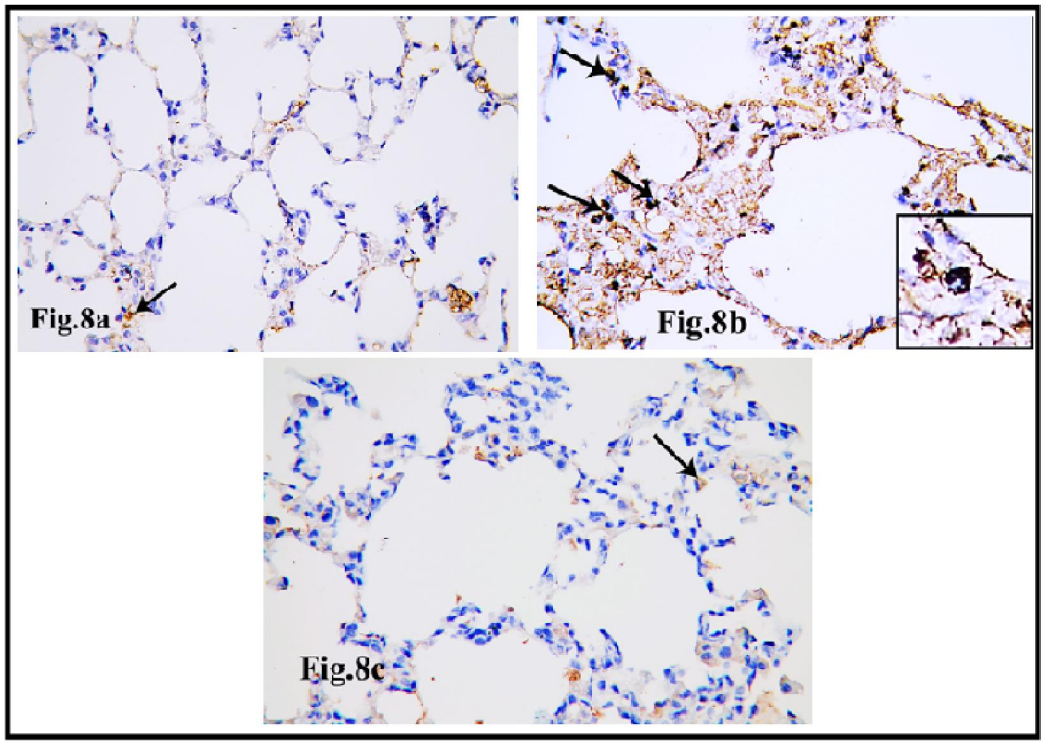


Figure 8: showing few CD68 positive cells (↑) in the alveolar septa of the control group (Figure: 8a). Aggregation of CD68 positive macrophage is seen in the alveolar septa (↑) of vitamin D_3 treated group (Figure: 8b). In simvastatin treated rats, few positive cells are seen (↑) (Figure: 8c). **CD68 X640**, **Inset X 1000**

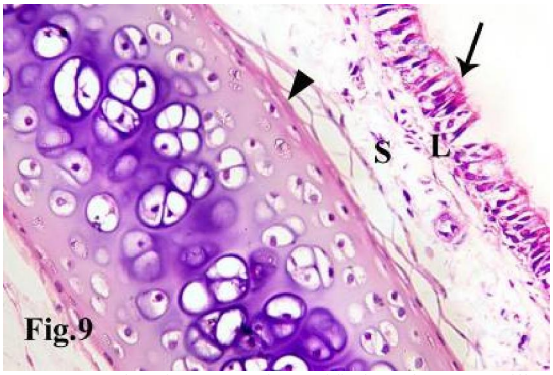


Figure 9: showing pseudostratified columnar ciliated epithelium with goblet cells (respiratory epithelium) (↑), lamina propria (L) and submucosa (S). Hyaline cartilage of the tracheal ring is seen covered with fibrous perichondrium (▲). Flattened chondrocytes, rounded chondrocytes, cell nests and basophilic matrix are also seen.
(Group I) H&E X 640

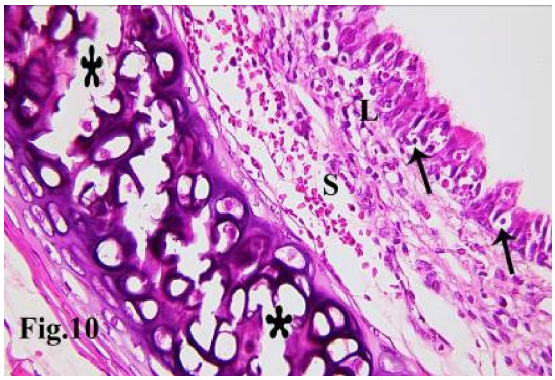


Figure 10: showing irregular festooned appearance of the epithelium with intra epithelial lymphocytes (↑). Cellular infiltration and extravasations of blood are seen in the lamina propria (L) and the submucosa (S). Notice irregularly fused empty chondrocyte lacunae (*) in the cartilaginous ring.
(Group II) H&E X 640

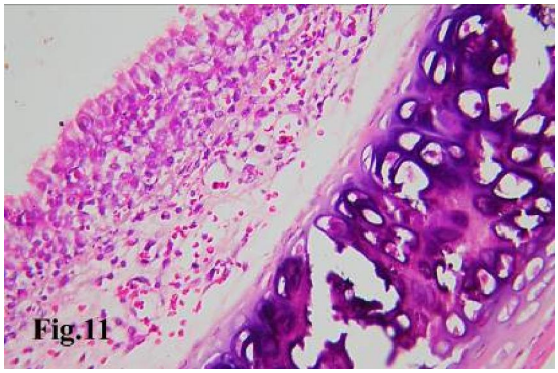


Figure 11: showing loss of demarcation between the epithelium and lamina propria. Cellular infiltration and extravasations of blood is also seen in the lamina propria and submucosa. **(Group II) H&E X 640**

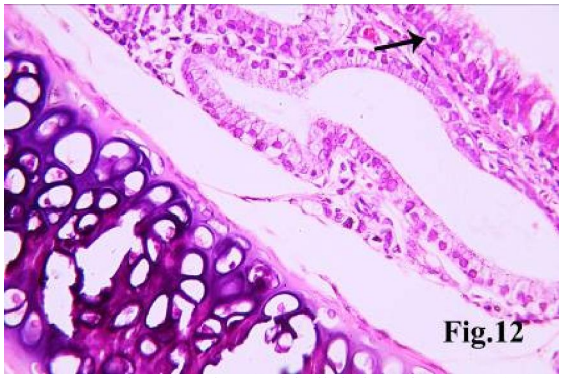


Figure 12: showing intraepithelial lymphocytes (↑) and hypertrophy of mucous glands in the submucosa.
(Group II) H&E X 640

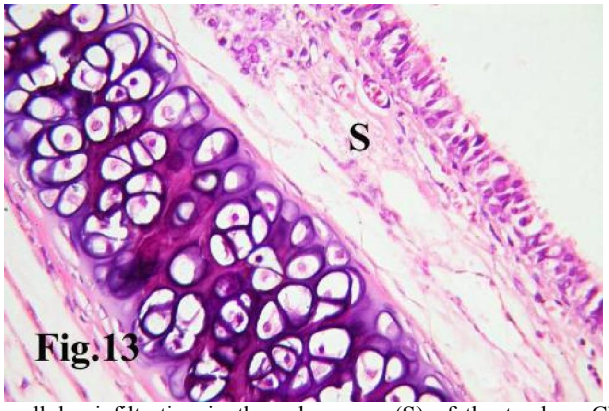


Figure 13: showing minimal cellular infiltration in the submucosa (S) of the trachea. Chondrocytes are seen in their lacunae. **(Group III) H&E X 640**

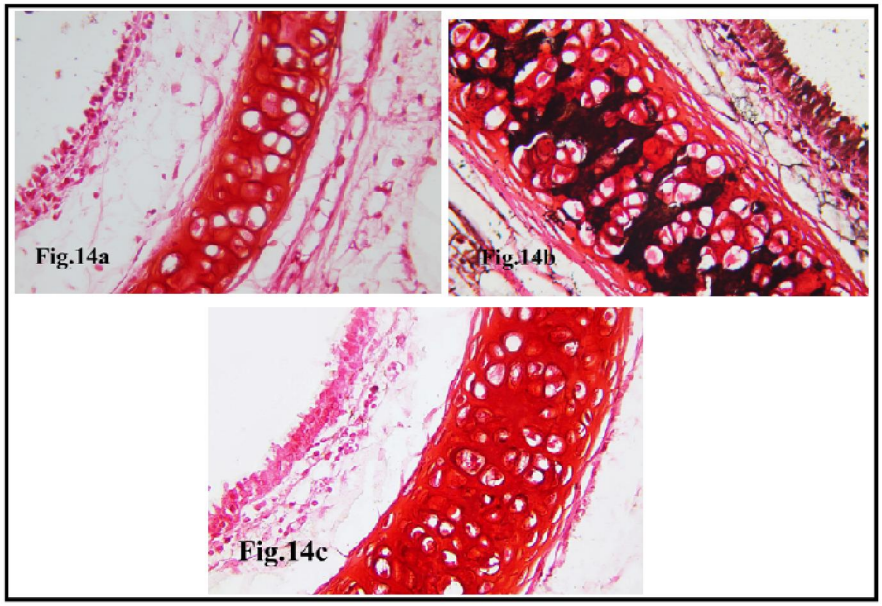


Figure 14: showing no brown deposits of calcium in the hyaline cartilage of the control group (Figure: 14a). Calcium deposition is seen in the matrix of the cartilage in vitamin D_3 treated group (Figure: 14b). No calcium deposits are seen in the hyaline cartilage of simvastatin treated group (Figure: 14c). **Von kossa X640**

The aim of the present study was to examine the effect of high dose vitamin D_3 on the structure of lung and trachea in adult male albino rats, and to examine the possible protective role of simvastatin on the developed changes**.**

In the current study, administration of high dose vitamin D_3 resulted in deposition of calcium in the lung interstitium. Similar observation was previously noticed by other investigators [6]. Deposition of calcium might lead to recruitment of inflammatory cells and macrophages through the wall of blood vessels to the lung interstitium, which was supported by the significant increase in CD68 positive macrophages. It was reported that acute insult resulted in release of proinflammatory mediators as interleukin 8 (IL8) that attract neutrophiles and macrophages. Activation of macrophages led to release of IL1 and tumor necrosis factor alpha (TNF-α) [19].

Extravasation of blood was frequently seen in lung interstitium in the current study. This might be explained by several mechanisms. It was reported that activation of endothelium by proinflammatory mediators (IL1, IL8 and TNF- α) resulted in endothelial contraction by histamine, expression of adhesion molecules and production of cytokines, chemokines and growth factors. Contraction of endothelium led to formation of intercellular gaps. Also leuckotriens released by activated neutrophils could attach to specific receptors on the endothelium causing changes in the cytoskeleton which cause prolonged retraction of endothelial cells. Other explanation reported that influx of calcium across plasma membrane led to increase cytosolic calcium which could activate several enzymes as phospholipase that result in membrane damage and proteases that break down the cytoskeletal protein [19]. The activity of protease and phospholipase could result in rupture of blood capillaries. In the current study, leucocytes were also seen adherent to the vessel wall. It was reported that accumulation of leucocytes along the vessel wall led to damage of endothelium which could result in vascular leakage [19].

It was reported that the activated neutrophils (by proinflammatory mediators), release toxic oxidants, proteases and leuckotriens. Release of these mediators could cause damage of endothelium and epithelium. Damage of epithelium led to loss of surfactant that renders the alveoli unable to expand. It was also reported that under the influence of proinflammatory cytokines (TNF- α, IL1 and IL8), neutrophils undergo sequestration in the pulmonary circulation and enter the alveolar space causing alveolar collapse [19]. These might be the causes of collapsed alveoli seen in the current study and the observation of dilated alveoli might be a compensatory mechanism.

It was reported that atelectasia is loss of lung volume caused by inadequate expansion of air spaces. Microatelectasia is present in lung diseases associated with interstitial inflammation and this type is potentially reversible and should be treated promptly to prevent hypoxemia and superimposed infection. It was reported that Accumulation of inflammatory cells in lung interstitium is called alveolitis. If the injury is

mild, resolution with restoration of normal architecture follows [19].

In the current study, administration of high dose vitamin D_3 resulted in hypertrophy of epithelial lining of bronchi, together with hyperplasia of goblet cells associated with prominent bronchus associated lymphatic nodules. It was reported that persistent inflammation of bronchi is manifested by the presence of inflammatory cells, hyperemia and increase number of goblet cells in the epithelium [19]. Increased goblet cells and formation of mucous layer on the surface of the bronchial epithelium might be a compensatory mechanism due to development of bronchitis.

Regarding the trachea, intraepithelial inflammatory cells were frequently noticed in the respiratory epithelium which was seen irregular and festooned. Also loss of demarcation was noticed between the epithelium and the lamina propria. Lamina propria and submucosa appeared heavily infiltrated with mononuclear cellular infiltration and extravasations of blood. Hypertrophy of submucosal mucous glands was also noticed. It is postulated that irritation of respiratory epithelium results in transcription of the mucin gene that led to enlargement of mucus secreting glands, increase number of goblet cells with concomitant loss of ciliated epithelium [19]. Deposition of calcium was also noticed in the current study in the matrix of hyaline cartilage of tracheal rings and chondrocyte lacunae were seen irregularly fused and empty. It was reported that chondrocytes normally take their nutrients and dispose wastes by diffusion of materials through the matrix. When the matrix becomes heavily calcified, diffusion is impeded and the chondrocytes swell and die [20].

In the current study, administration of simvastatin before giving high dose vitamin D_3, attenuated changes that occurred in both lung and trachea. A significant reduction in the mean area percentage of CD68 expression was noticed in rats pretreated with simvastatin. It was previously reported that statins inhibit macrophage infiltration [21] and could induce apoptosis of macrophages [22]. It was reported that lung injury is caused by imbalance between proinflammatory and anti-inflammatory cytokines [19]. And statins was known to have antioxidants effects [23] and could suppress the production of inflammatory molecules [23, 24]. It was reported that the destructive force of neutrophils can be counter acted by endogenous antioxidants, antiproteases and anti-inflammatory cytokines. The balance between destructive force and protective factor determine the degree of tissue injury [19]. As statins have several protective effects as anti-inflammatory, antioxidant effects as well as causing apoptosis of macrophages. All these effects help in the protective function of simvasatatin in hypervitaminosis D_3 induced structural changes in both lung and trachea. This may support the hypothesis that the positive effects of statins extend beyond its cholesterol-lowering properties [25].

Conclusions:

In the current study, administration of high dose vitamin D_3 produced metastatic calcification, mononuclear cellular infiltration, extravasations of blood and hyperplasia of mucous secreting cells in both lungs and trachea with thickening of interalveolar septa. Pretreatment with simvastatin, ameliorated these changes.

Recommendations:

Vitamin D administration implies several risks and must be prescribed only when needed and under strict medical control. Patients with conditions that predispose to hypercalcemia should be monitored carefully. Prophylactic administration of statins could be used in these patients.

Acknowledgments

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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