Statins-Induced Lung Toxicity and Its Possible Molecular Mechanisms in Adult Albino Rats

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Abstract: Statins have become the most prescribed lipid lowering drugs during the last years. They recently used in the treatment of dyslipidemia, and ischemic heart diseases, and in the prevention of stroke, atheromatous diseases and Alzheimer's disease. Their toxic effects on the lung haven't been studied extensively, and its molecular, mechanisms still need more clarifications. The aim of the current work was to study lung toxicity induced by Simvastatin and Atorvastatin as being the most commonly used statins and to investigate possible underlying molecular mechanisms of such toxicity in adult albino rats. Twenty eight female albino rats were divided equally into 4 groups: group I (negative control), group II (positive control): daily received 2 ml of distilled water, group III (simvastatin treated group): daily received toxic dose of Simvastatin, 200 mg/kg b.wt. for 3 weeks, group IV (Atorvastatin treated group): daily received toxic dose of Atorvastatin 250 mg/kg b.wt. for 3 weeks. Both of Simvastatin and Atorvastatin treatment showed significant decrease in the level of total cholesterol, triglycerides, low density lipoproteins, very low density lipoproteins, (P<0.01), and a significant increase in the levels of high density lipoproteins (P<0.01), and endothelial nitric oxide activity (P<0.05), with a marked toxicological cellular changes as compared with control group. Neither Simvastatin nor Atorvastatin induced expression of the Caspase-3. It was concluded that administration of statins induces toxic effects on the normal lung tissues through lipid lowering dependent and non-dependent mechanisms specially, the Nitric Oxide dependant one.
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Keywords: Simvastatin, Atorvastatin, Phospholipidosis, Apoptosis, Caspase-3 and eNOS.

1. Introduction

Statins, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors are hypolipidemic drugs used to control hypercholesterolemia. They have become the most commonly prescribed agents for the treatment of dyslipidemia (Wang et al., 2001). Statins are also being recognized to have potential application in peripheral arterial disease, end-stage renal disease, diabetes mellitus, and Alzheimer's disease (McKenney, 2003; Vega *et al.,* 2003). In animal studies, they also appear to be beneficial in traumatic brain injury (Lu *et al.,* 2004) and neuroinflammation (Adamson and Greenwood, 2003).

Andrews *et al.,* (2001) stated that Atorvastatin and Simvastatin are the two statins that produce the greatest percentage change in the Low Density Lipoprotien cholesterol (LDL).

Simvastatin is a semi synthetic derivate of a fermentation product of Aspergillus terreus fungus. It is widely used clinically for the prevention of atheromatous diseases. Moreover, it reduces the risk of ischaemic heart disease events by 60% and stroke by 17% (Law *et al.,* 2003).

Atorvastatin is a synthetic statin compound. The drug has topped the list of best-selling pharmaceuticals in the world for nearly a decade until 2010 (Jin, and Ulrich, 2010).

The toxic effects of statins on musculoskeletal, neurologic and, hepatic tissues have been studied extensively (Baselt, 2008). However, studies concerning with statins-induced lung toxicity are very limited. Furthermore, its underlying molecular mechanisms need more clarification. Phospholipidosis, apoptosis and Nitric Oxide dependant mechanisms are described as essential mechanisms of action of statins. The current work was designed to test capability of statins to induce lung toxicity, and to test which of the previous molecular mechanisms could be responsible of such toxicity.

Phospholipidosis is a phospholipid storage disorder, induced by the short-term administration of cationic amphiphilic drugs (CADs). The metabolism of this essential cell component is regulated by the individual cell and may be altered by drugs that interact with phospholipids or the enzymes that affect their metabolism. Xenobiotics or their metabolites that induce phospholipidosis include a wide variety of pharmacologic agents as antipsychotics, antidepressants, antiarrhythmics, antianginals, anorexic agents and cholesterol-lowering agents, including statins. Each of these drugs shares several common physiochemical properties with a charged cationic amine group, hence the class term CADs. Although phospholipidosis may

occur in almost all tissues in the body, the lung and the alveolar macrophages are usually prominent in their response to administration of most CADs (Halliwell, 1997 and Baselt, 2008)

Apoptosis, a process of programmed and regulated cell death, is an active energy-dependent process in which a cell commits itself to an organized program of self-destruction (Hengartner, 2000). Apoptosis has currently gained central importance in modern biology. Research activity in this area has expanded exponentially during the last two decades (Gulbins *et al.,* 2003). Casepase- 3 is a critical executioner of apoptosis, as it is either partially or totally responsible for the proteolytic cleavage of many key proteins. Recently, advances in the understanding of the molecular events in apoptosis have led to the realization that caspase activation is by far the most specific indicator of this cell suicide mechanism. Several publications have reported that caspase-3, a ubiquitously distributed caspase is a main effector caspase of the apoptotic cascade within cells. They also suggested that it could be a highly specific and sensitive method generally applicable to many studies (Gown and Willingham, 2002). Moreover, Duan *et al.* (2003) indicated that activated caspase-3 immunohistochemistry is an easy, sensitive, and reliable method for detecting and quantifying apoptosis.

Nitric oxide (NO) is produced by a group of enzymes called nitric oxide synthases (NOS). These enzymes catalyze the production of NO and L-citrulline from L-arginine, O_2, and NADPH-derived electrons. Mammalian systems contain 3 isoforms of NOS: neuronal NOS, inducible NOS, and endothelial NOS (eNOS). The eNOS is expressed in vascular endothelial cells and cardiomyocytes. NO produced by the eNOS in the vascular endothelium, yields a labile intercellular messenger molecule with potent biological activities including vascular smooth muscle relaxation. Expression of eNOS is usually reported to be constitutive (Busconi and Michel, 1993).

Aim of the work

The aim of the current work was to study lung toxicity induced by Simvastatin and Atorvastatin as being the most commonly used statins and to investigate the possible underlying molecular mechanisms of such toxicity in adult albino rats.

2. Material and Methods

Twenty eight adult male albino rats, weighing 200-250 g were used in this study. They were purchased from the animal house of Zagazig Faculty of Medicine. All rats were housed in stainless-steel cages and the laboratory conditions were kept optimal in a controlled environment, to standardize the experiment's condition.

Experimental Design: The rats were divided equally into 4 groups (each of 7 rats): **group I** (negative control), **group II** (positive control): daily received 2 ml of distilled water, **group III** (simvastatin treated group): daily received oral dose of Simvastatin, 200 mg/kg b.wt. (1/20 of LD_{50}) (Yakuri, 1990) for 3 weeks, **group IV** (Atorvastatin treated group): daily received oral dose of Atorvastatin 250 mg/kg b.wt. (1/20 of LD_{50}) (Henck *et al.,* 1998) for 3 weeks.

Drugs and chemicals:

*Simvastatin was obtained in the form of white powder from Global Napi Pharmaceuticals (GNP), with a license from Merck and Co., Inc.

*Atorvastatin was obtained from Pfizer Inc. Ltd., Pfizer Pharmaceuticals Group.

* Caspase-3: Rabbit Polyclonal antibody (CPP32 Ab-4) Cat. No. RB-1197 was obtained from Thermo Fisher Scientific, USA.

Both drugs were freshly suspended in distilled water and given to rats through oral gavage.

* eNOS Enzyme kit: Rat Endothelial Nitric Oxide Synthase (eNOS) ELISA Kit Cat No. CSB-08323r was obtained from GENTAUR Molecular Products Ltd, Belgium.

Methods:

*Lipid Profiles: Colorimetric assessment (570nm) was done by following the methods described by Henry *et al.,* 1974. Blood samples (2ml) were collected from the retro orbital plexus with the help of capillary tubes, centrifuged to separate serum using standard plain tubes. Total Serum Cholesterol (TC) (Deeg and Ziegenohrm, 1983), Triglycerides (TG) (Fossati and Prencipe, 1982), Low Density Lipoprotein Cholesterol (LDL), Very Low Density Lipoprotein Cholesterol (VLDL) and High Density Lipoprotein Cholesterol (HDL) (Friede *et al.,* 1972) were assessed. Serum LDL and VLDL were estimated by calculation based on Friedwald *et al.,* 1972. Where: LDL mg/dl = Total cholesterol – HDL - TG/5. VLDL mg/dl = Serum triglycerides 5

*Endothelial Nitric Oxide Synthase Enzyme (eNOS) assay: eNOS activity was assayed by ELISA technique according to the manufacturing procedure guideline (Wood, 1991). Blood samples of 2 ml were collected and allowed to clot for 30 minutes before centrifugation for 15 minutes at approximately 1000×g and stored at -80ºC. The provided microtiter plate has been pre-coated with antibody specific to eNOS. Samples were added to the microtiter plate wells with a biotin-conjugated polyclonal antibody preparation specific for eNOS. Avidin conjugated to Horseradish Peroxidase (HRP) was added to each microplate well and incubated. Then a TMB (3,3',5,5' tetramethyl-benzidine) substrate solution was added to each well. Only those wells that contain eNOS, biotin-conjugated antibody and enzyme-conjugated

Avidin exhibited a change in color. The enzyme-substrate reaction was terminated by the addition of a sulphuric acid solution and the color change was measured spectro photometrically at a wavelength of 450 nm. The concentration of eNOS in the samples was determined by comparing the optical density (O.P.) of the samples to the standard curve and expressed as IU/ml (Meurer *et al.,* 2002).

*Light microscopic examination of lung tissues: Paraffin blocks and sections were prepared, by fixing lung specimens in 10% neutral formol saline for 24 hrs and were processed to prepare 5 µm-thick paraffin sections and stained with hematoxylin and eosin to verify the histological details, by following the method of (Bancroft and Gamble, 2002).

* Ultrastructure Study: According to the method described by Glauert and Lewis, 1998, specimens of the lungs were immediately fixed in 2.5% glutaraldehyde buffered with 0.1 mol/l phosphate buffer at a pH of 7.4 for 2 hrs and then post fixed in 1% osmium tetroxide in the same buffer for 1 h. They were processed to prepare semithin sections and then ultrathin sections. Ultrathin sections were obtained using a Leica ultracut UCT (Germany) and stained with uranyl acetate and lead citrate. They were examined with a JEOL JEM 1010 electron microscope in the Electron Microscope Research Laboratory of the Histology and Cell Biology Department, Faculty of Medicine, Zagazig University, and with a JEOL JEM 1200 EXII electron microscope at the Faculty of Science, Ain Shams University, Egypt.

*Immunohistochemistry of caspase-3: caspase-3 immunohistochemistry was performed by following the method of Ramos-Vara *et al.,* 2008. The paraffin sections were processed by Streptavidin-biotin complex (Strep ABC). The sections of 4 µm paraffin sections were deparaffinized in xylene and rehydrated in a descending series of ethanol. The specimens were subjected to antigen retrieval in citrate buffered solution (pH 6.0) for 10 min by microwave. Endogenous peroxidase was eliminated by incubation in 10% H_2O_2 in phosphate-buffered saline (PBS; pH: 7.4) for 10 min. After being washed, the specimens were blocked in ready-use normal goat serum for 20 min at room temperature. The sections were incubated with caspase3 antibody. Primary antisera were diluted in antibody diluent (Labvision, TA-125-UD). The peroxidase activity was demonstrated by AEC (3- amino-9-ethyl carbazole) substrate kit (Labvision, TA-004-HAC). The sections were rinsed in phosphate buffered saline (PBS). Negative control was obtained when the primary antibody was replaced with PBS (Gown and Willingham, 2002).

3. Results

On comparing the results of lipid profile parameters in negative control group (TC 89.67±3.77 mg/dl, TG 65.33±1.52 mg/dl**,** HDL 24±1.93 mg/dl, LDL 52.60±3.66 mg/dl, VLDL 13.06±0.30 mg/dl and eNOS 53.34±12.36 IU/ml) and positive control group (TC 90.67±1.52 mg/dl, TG 65.67±1.45 mg/dl, HDL 26.83±1.38 mg/dl, LDL 50.57±2.58 mg/dl, VLDL 15.13±0.29 mg/dl, 2.73±0.20 mg/dl and eNOS 53.35±12.36 IU/ml), there was a non significant difference as shown in **table (1) and fig. (I a, b).** Light microscopic and transmission electron microscopic examination of lung tissues in the control rats showed normal architecture of alveoli, alveolar sacs, and interalveolar septa. The lining epithelium of the alveoli composed of squamous cells (type I pneumocytes) and large cuboidal cells (pneumocytes type II) **(Figs. 1-4).** Caspase-3 expression of the examined specimens was negative **(Fig. 5).**

Simvastatin treated group showed significant decrease in the level of total cholesterol 74.83±3.76, TG 55.17±1.72, LDL 27.30±5.58, VLDL 11.03±0.34 (P<0.01), and a significant increase in the levels of HDL 36.50±2.79 (P<0.01), and eNOS activity 84.38±11.72 (P<0.05), as compared with control group **Table (1)** and **Fig. (I $_{a, b}$).**

On the other hand, it was found that simvastatin administration induced histological changes in the lung tissues. Pneumocytes type I had a flat euchromatic nucleus and separated by thin interalveolar septa. Pneumocytes type II showed large rounded euchromatic nucleus and short microvilli on the cell surface with a cytoplasmic lamellar bodies.

Transmission electron microscopic examination showed focal areas of collapsed alveoli with compensatory dilatation of neighboring ones separated by relatively thick interalveolar septa. Congested blood vessels, cellular infiltration with moderate inflammatory cellular infiltration of interalveolar septa were observed. Extravasated red blood cells with foamy cytoplasmic interstitial cells were also noticed. Pneumocytes type II showed degenerative changes of their lamellar bodies leaving irregular empty vacuoles and apical characteristic microvilli. Immuno histochemical study of the caspase-3 reaction showed negative reaction (**Figs 5-13**).

Atorvastatin treated group showed significant decrease in the level of total cholesterol 72.53+2.98, TG 53.37+1.25 and LDL 15.33+2.69, VLDL 12.20+0.97 (P<0.01), with a significant increase in the levels of HDL 40.0±2.16 (P<0.01) and eNOS activity 87.38±11.72 (P<0.05) **Table (1)** and **Fig. (I a, b).**

Examination of lung tissue specimens of Atorvastatin treated group showed collapsed alveoli lined by many

electron-dense pneumocytes type II with degenerative changes of their lamellar bodies leaving irregular empty vacuoles. An intra-alveolar macrophage with pseudopodia and interstitial collagen fibers are also observed. Collapsed alveoli, lined by electron-dense pneumocyte type II with vacuolated lamellar bodies were observed. Exfoliated pneumocyte type II with vacuolated lamellar bodies and few characteristic lamellar bodies with mild thickening of interalveolar septa and congested blood capillaries were also detected. Ultrastructure examination showed alveolar macrophage with indented nucleus, long pseudopodia and numerous cytoplasmic lysosomes. Marked increase in collagen fibers deposition within the interalveolar septa was also observed. Negative Caspase-3 reaction was noticed in all specimens **(Figs. 14-21).**

Comparison of lipid profile results and eNOS activity in Simvastatin-treated group and Atorvastatin-treated group showed a non significant difference as noticed from **table (1),** while morphological changes of the lung tissues induced by Atorvastatin administration were more extensive than Simvastatin as noticed in **Figs. 5-19.**

Both drugs did not produce apoptosis through caspase-3 mediated pathway as detected by the presence of negative caspase-3 reaction in all examined specimens **(Figs 7, 17).**

4. Discussion

The current study showed that Simvastatin and Atorvastatin induced significant effects on lipid profile parameters. These results were in a harmony with all previous data about the beneficial effects of statins group of pharmaceutical. They stated that despite differences in their pharmacokinetic profiles, all statins have at least one characteristic in common: they block the conversion of HMG-CoA to mevalonic acid, the rate-limiting step of cholesterol synthesis, with consecutive attenuation of the biosynthesis of cholesterol, which is associated with a reduction in serum total and low-density lipoprotein (LDL) cholesterol. Because of these properties, statins have become the most widely prescribed lipid-lowering drugs in patients with elevated serum cholesterol levels. Another pathway affected by statins seems to be the regulation of the activity of the enzyme cholesteryl ester transfer protein (CETP), which transfers cholesteryl ester to very-low-densitylipoprotein (VLDL) and LDL. It was found that statins decrease plasma CETP activity in normolipidemic individuals and patients with various forms of hyperlipoproteinemia (Napoli *et al.,* 1998; Istvan and Deisenhofer, 2001 and Bonetti *et al.,* 2003.

On the other hand, It was found that the differential effects between Simvastatin and Atorvastatin in lowering TC, TG, LDL and VLDL were non significant. These results were consistent with the findings of McKenney *et al.,* 2003 and Nicholls *et al.,* 2010 when comparing the efficacy of rosuvastatin, atorvastatin, simvastatin and paravastatin in achieving lipid goals.

The present study showed that both of Simvastatin and Atorvastatin induced toxic changes in the structure of lung tissues, when examined by the light and electron microscopes.

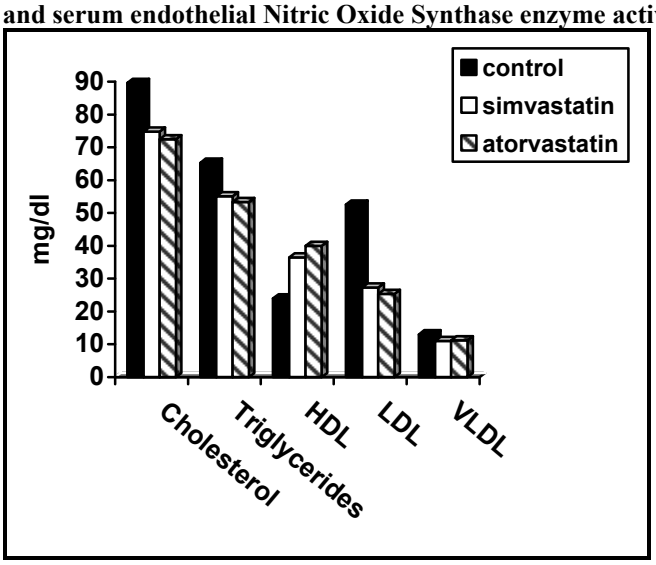
The previous findings could be explained by the study of Halliwell, 1997. The author stated that drugs containing a cationic lipophilic structure are capable of inducing phospholipidosis in cells. That is because this type of structure typically contains a hydrophilic domain consisting of one or more primary or substituted nitrogen groups. Those groups are positively charged at physiological PH. Additionally, hydrophobic domain is consisting of an aromatic and/or aliphatic ring structure, which may be substituted with one or more of halogen moieties. This class of drugs has been historically referred to as cationic amphiliphilic drugs. Phospholipidosis induced by the drugs with a cationic amphiphilic structure, is a generalized condition in humans and animals. This condition characterized by an intracellular accumulation of phospholipids and the concurrent development of concentric lamellar bodies.

Reasor and Kacew, 2001 stated that Phospholipidosis is a cellular process caused by excessive formation and accumulation of intracellular phospholipids. Lipid accumulation leads to the formation of abnormal lamellated inclusion bodies, which are considered characteristic features of this process. The primary mechanism responsible for the development of phospholipidosis is an inhibition of lysosomal phospholipidase activity by the drugs. While the biochemical and ultrastructural features of the condition have been well characterized, much less effort has been directed toward understanding its mechanisms.

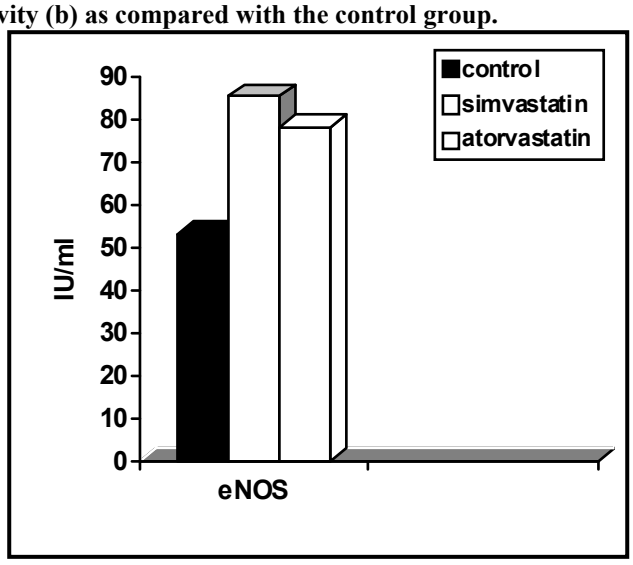
Cationic amphiphilic drugs have been shown to cause phospholipidosis in animal and human cell cultures by inactivating phospholipase C-g1 (PLC-g1). PLC-g1, a tyrosine kinase widely distributed in bronchiolar epithelial cells and fibroblasts, regulates cell proliferation and differentiation. Activated PLC-g1 hydrolyses phosphatidylinositol- bisphosphate (PIP2) into inositol 1,4,5-trisphosphate and diacylglycerol; inositol 1,4,5-trisphosphate regulates intracellular calcium levels, while diacylglycerol activates protein kinase C, which is involved in the control of apoptosis. (Manzoli *et al.,* 2005; Ernawati *et al.,* 2008 and Papiris *et al.,* 2010).

Regarding apoptosis, the current study showed that both of Simvastatin and Atorvastatin induced negative reaction of caspase-3, as a marker of apoptosis, in all examined lung tissues.

Plot (I): Bar charts showed significant effects of Simvastatin and Atorvastatin on lipid profile parameters (a) and serum endothelial Nitric Oxide Synthase enzyme activity (b) as compared with the control group.



(a)



(b)

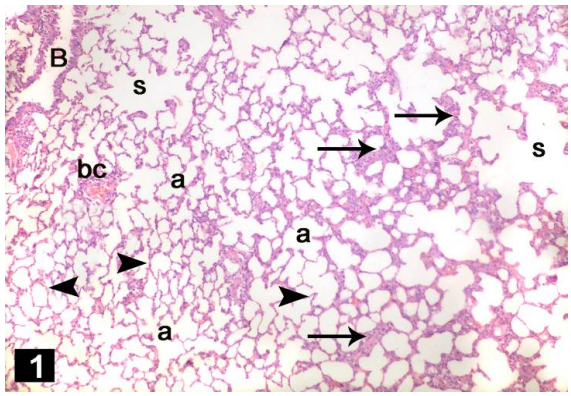


Fig. (1): A photomicrograph of a section in rat lung of control group showing a normal lung architecture with alveoli (a), alveolar sacs (s), thin (arrow heads) and thick (arrows) portions of interalveolar septa. A bronchiole (B) and blood vessels (bc). *H& E, × 100.*

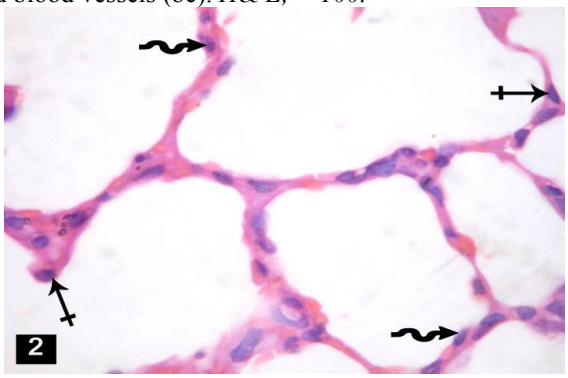


Fig. (2): A photomicrograph of a section in rat lung of control group showing the lining epithelium of alveoli composed of squamous cells (type I pneumocytes) (wavy arrow) and large cuboidal cells (pneumocytes type II) (crossed arrow). *H&E, × 400.*

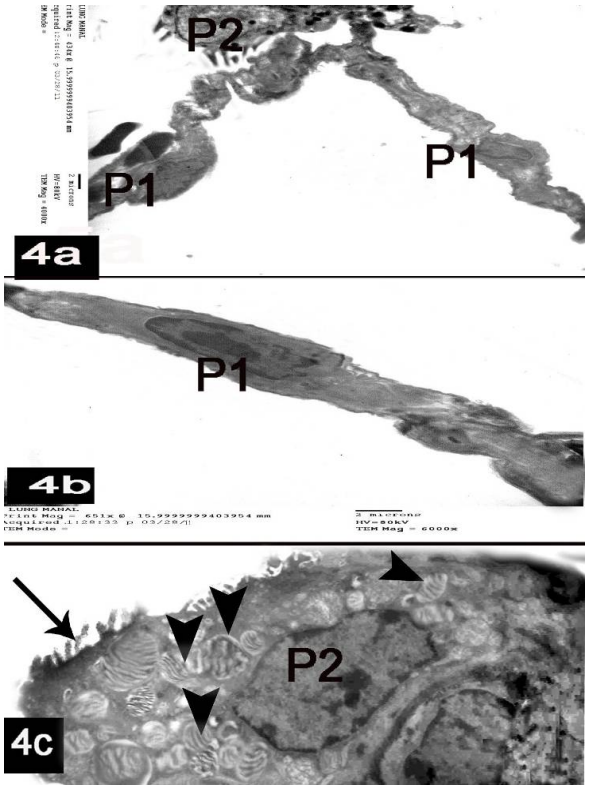


Fig. (4): An electron micrograph of a section in rat lung of control group (a) showing parts of adjacent alveoli lined by pneumocytes type I (P1) and a pneumocytes type II (P2), (b) showing a pneumocytes type I, flat euchromatic nucleus (P1), and (c) showing a pneumocytes type II having large rounded euchromatic nucleus (P2) and short microvilli on the cell surface (arrows). Its cytoplasm shows lamellar bodies (arrow heads). *EM; a X4000, bX4000,c X5000*

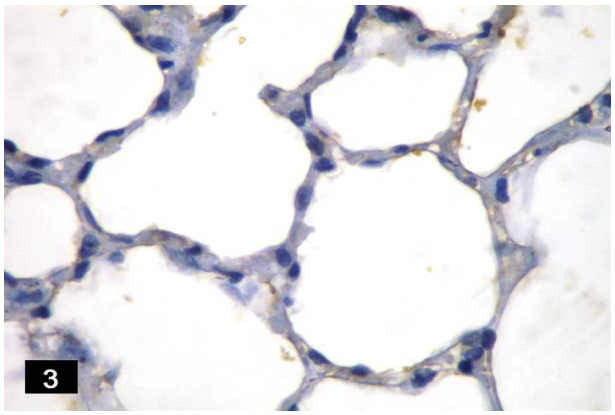


Fig. (3): A photomicrograph of a section in rat lung of control group showing a negative immune reaction in the alveolar lining cells. *Caspase 3 X 400* .

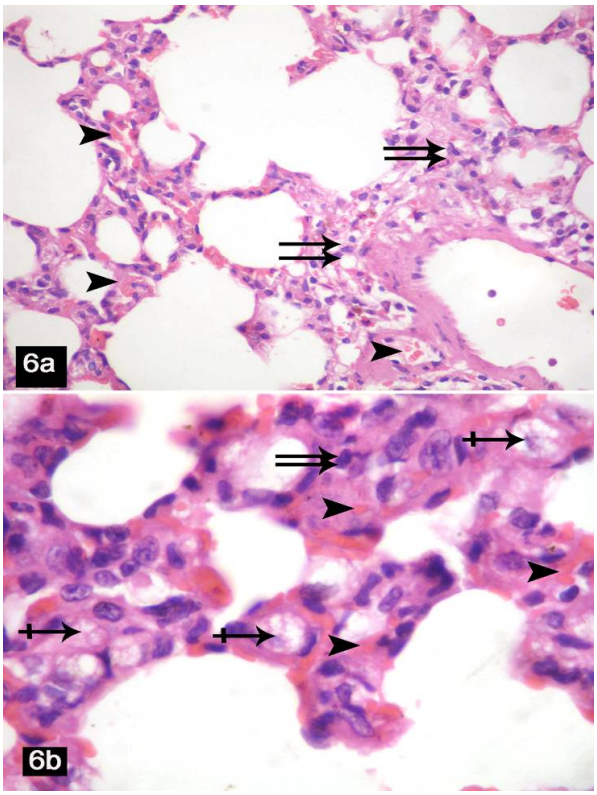


Fig. (6): A photomicrograph of a section in rat lung of group II (a) showing moderate inflammatory cellular infiltration (double arrows) in the interalveolar septa (s). Extravasated red blood cells (arrow heads) are noticed. (b) showing inflammatory cellular infiltration (double arrows), extravasated red blood cells (arrow heads) and interstitial cells with foamy cytoplasm (crossed arrows). *H&E, (a) × 400 (b) X1000.*

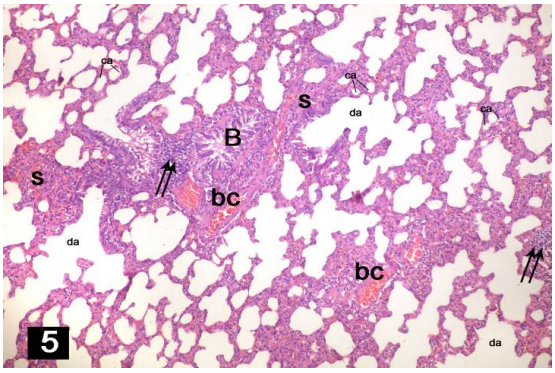


Fig. (5): A photomicrograph of a section in rat lung of group II showing focal areas of collapsed alveoli (ca) with compensatory dilatation of neighboring ones (da) separated by relatively thick interalveolar septa (s). Congested blood vessels (bc), cellular infiltration (double arrow) and a bronchiole (B) are noticed. *H&E, × 100*

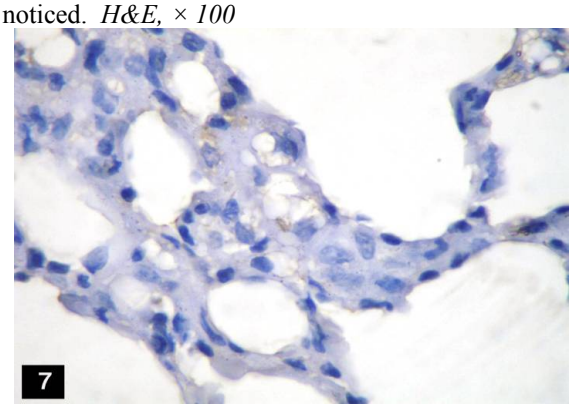


Fig. (7): A photomicrograph of a section in rat lung of group II showing showing a negative immune reaction in the alveolar lining cells. *Caspase 3 X400.*

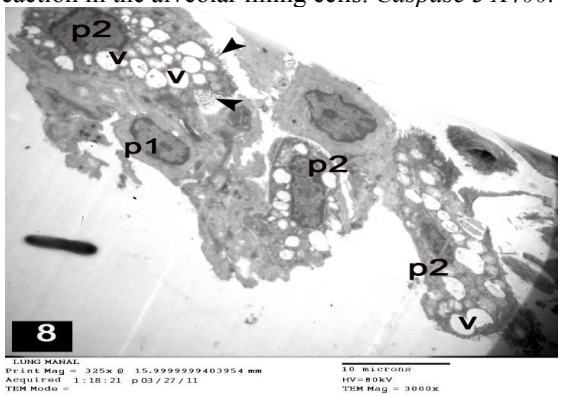


Fig. (8): An electron micrograph of a section in rat lung of group II showing alveoli (a) lined by pneumocytes type I (P1) and many pneumocytes type II (P2) with degenerative changes of their lamellar bodies leaving irregular empty vacuoles (v) and apical characteristic microvilli (arrow heads). *EM X3000*

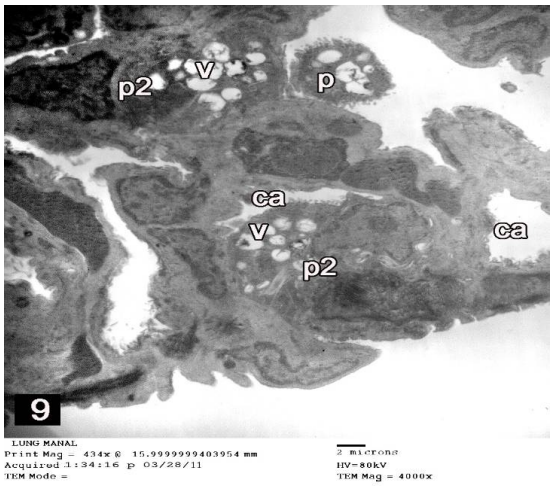


Fig. (9): An electron micrograph of a section in rat lung of group II showing collapsed alveoli (ca) lined by many electron-dense pneumocytes type II (P2) with degenerative changes of their lamellar bodies leaving irregular empty vacuoles (v). Detached pneumocyte type 2(P) in the interalveolar lumen. *EM X4000*

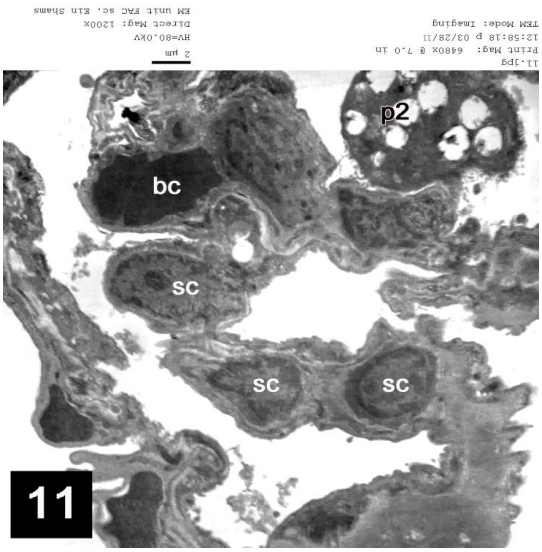


Fig. (11): An electron micrograph of a section in rat lung of group II showing many interstitial septal cells (SC). An exfoliated pneumocyte type II (P2) with vacuolated lamellar bodies and congested blood capillaries (bc). *EM X1200*

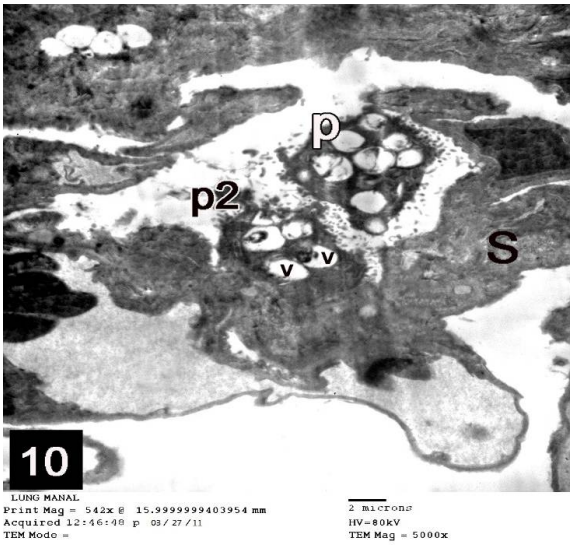


Fig. (10): An electron micrograph of a section in rat lung of group II electron-dense pneumocyte type II (P2) with vacuolated lamellar bodies (v). Another exfoliated pneumocyte type II (P) is also detected. Mild thickening of interalveolar septa (s). *EM X 5000*

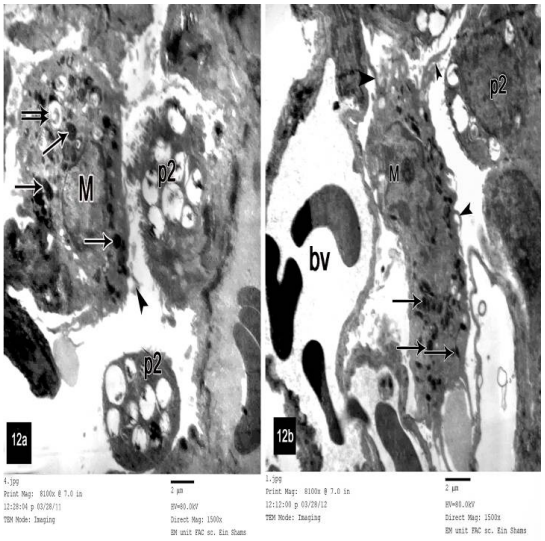


Fig. (12): An electron micrograph of a section in rat lung of group II showing alveolar macrophages (M) with long pseudopodia (arrow heads) and numerous cytoplasmic primary lysosomes (arrows) and secondary lysosomes (double arrows) are observed. Pneumocyte type II cells with vacuolated lamellar bodies are seen. *EM, a X1500, bX 1500*

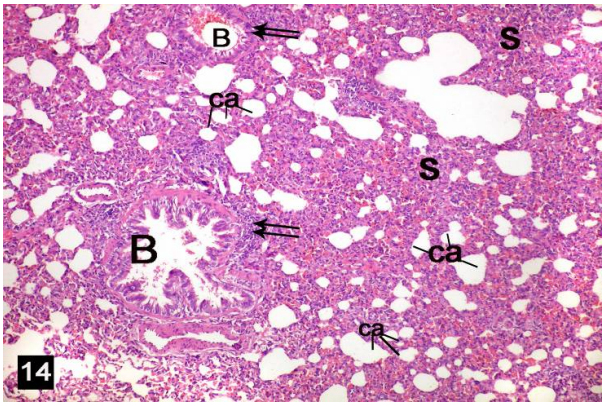


Fig. (14): A photomicrograph of a section in rat lung of group III showing marked thickening of interalveolar septa (s) with heavily interstitial inflammatory cellular infiltration (double arrows) especially around bronchioles (B) and Severely collapsed alveoli (ca). *H&E X100.*

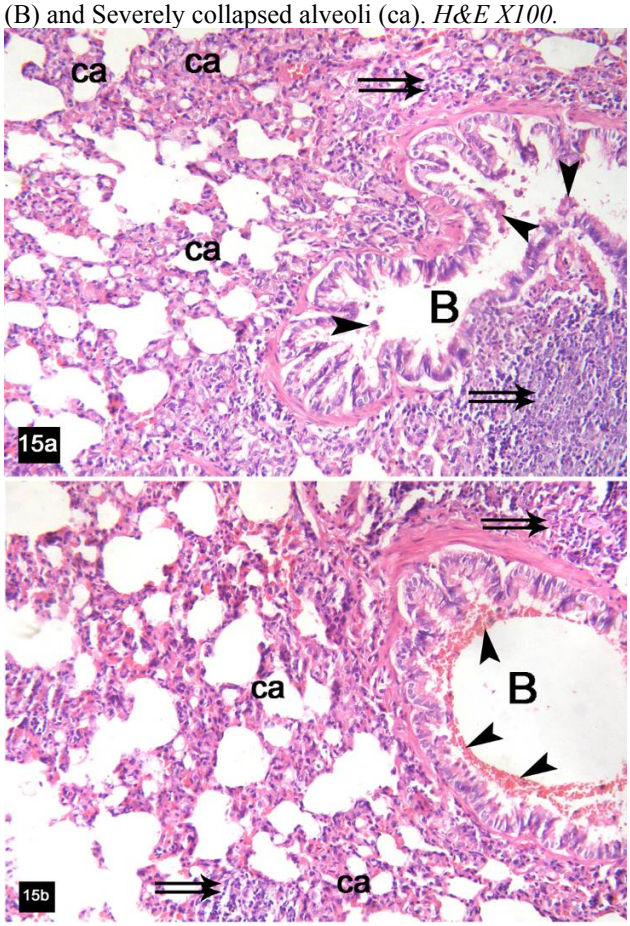


Fig. (15): A photomicrograph of a section in rat lung of group III (a) showing Intrabronchial cellular debris (arrow heads). Collapsed alveoli (ca) separated by thickened interalveolar septa and a pronounced inflammatory cellular infiltration (double arrows) are also observed. (b) showing intrabronchial (B) RBCs (arrowheads) with collapsed alveoli (ca) and inflammatory cellular infiltration (double arrows). *H&E a, b X 400.*

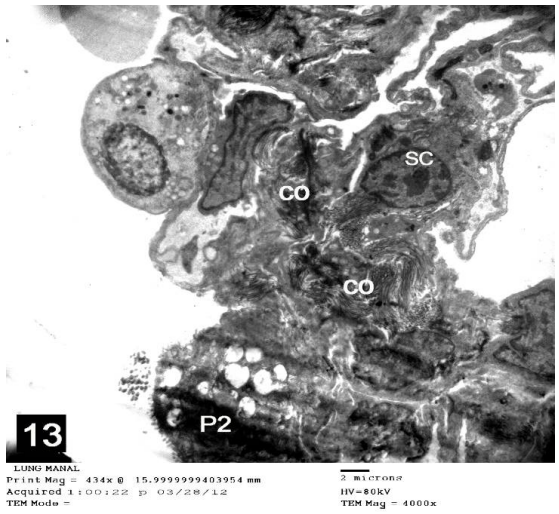


Fig. (13): An electron micrograph of a section in rat lung of group II showing marked increase in collagen fibers deposition (Co) within the interalveolar septa. Part of pneumocyte II (P2) and an intersticial cell (sc) are seen. *EM X4000*

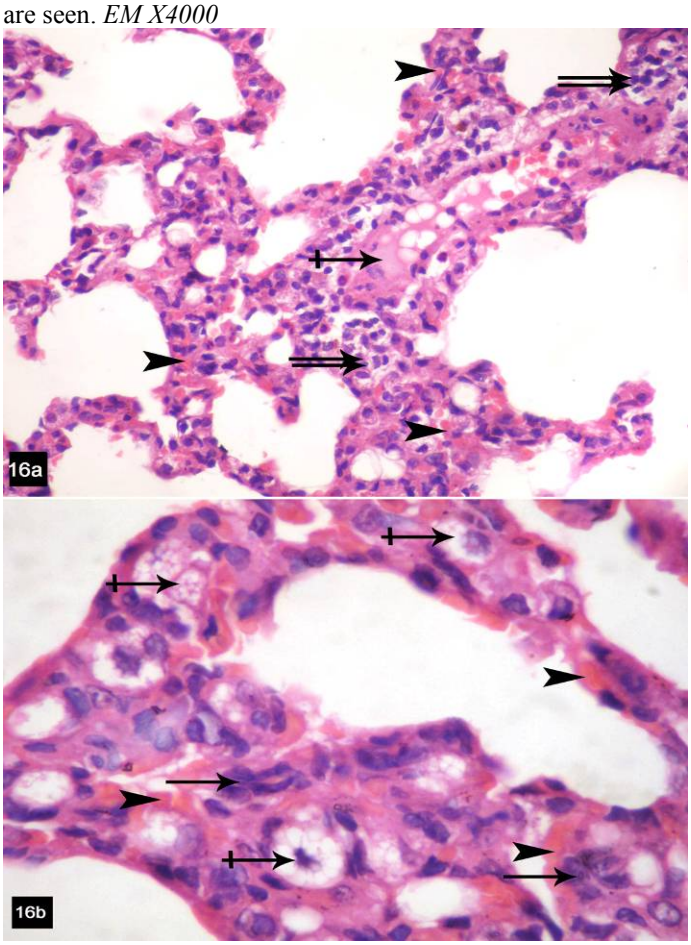


Fig. (16): A photomicrograph of a section in rat lung of group III (a) showing massive inflammatory cellular infiltration in the interalveolar septa (double arrows), extravascular RBCs (arrow heads) and

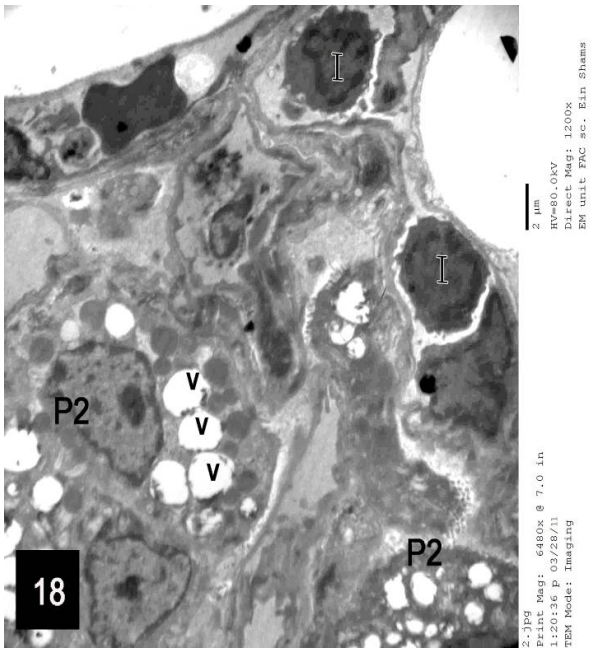


Fig. (18): An electron micrograph of a section in rat lung of group III showing many mononuclear inflammatory cells (I). Many pneumocytes type II (P2) are observed lining the wall of collapsed alveoli with many vacuoles like structure (v). *EM X1200*

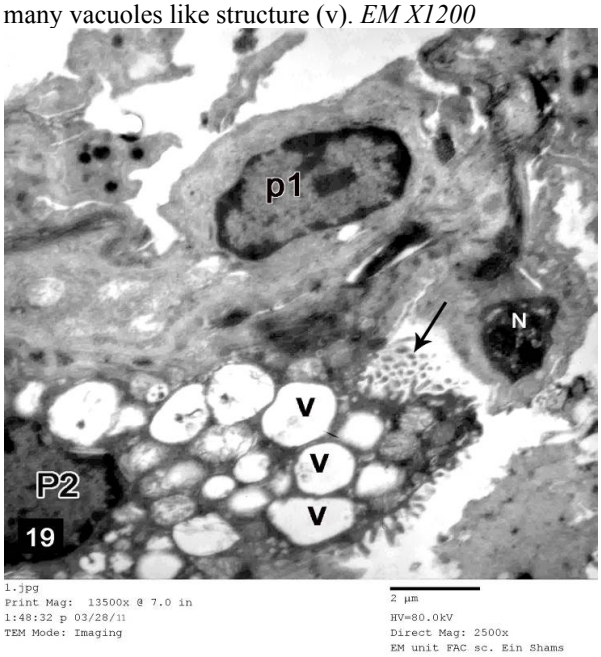


Fig. (19): An electron micrograph of a section in rat lung of group III showing An alveolar lining cell with small heterochromatic nucleus (N). a pneumocyte type I (P1) having a nucleus with peripheral margination of heterochromatin and pneumocyte type II (P2) with vacuolated lamellar bodies (v) and characteristics apical microvillar border (arrow). *EM X2500*

eosinophilic homogenous material (crossed arrows). (b) showing many septal large cells with foamy cytoplasm (crossed arrows) and extravascular RBCs (arrow heads). H*&E aX 400, bX 400.*

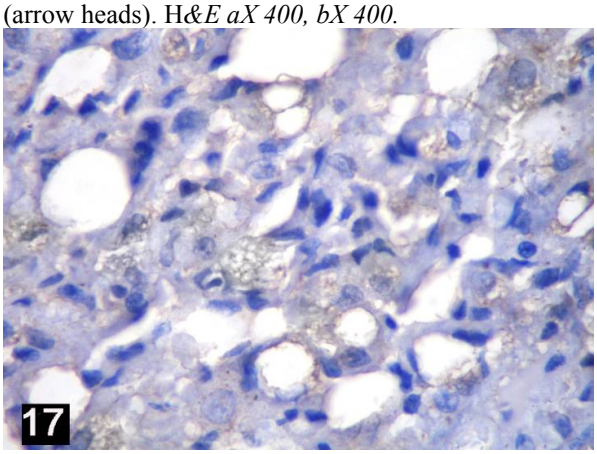


Fig. (17): A photomicrograph of a section in rat lung of group II showing showing a negative immune reaction in the alveolar lining cells. *Caspase- 3 X400*

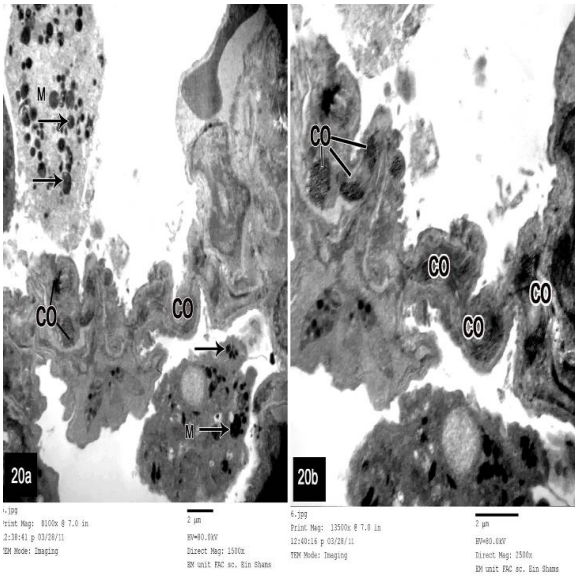


Fig. (20): An electron micrograph of a section in rat lung of group III (a) showing many collagen fibers deposition (co) within the interalveolar septa (s). intraalveolar macrophage cells (M) with many electron dense lysosomes (arrows). (b) higher magnification of the (fig. 20 a) *EM, a X1500, bX2500*

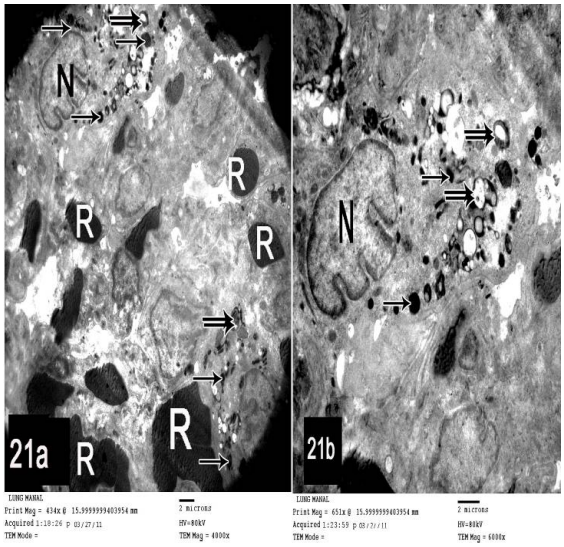


Fig. (21): An electron micrograph of a section in rat lung of group III (a) showing interstitial cells with large indented nuclei (N) and many electron dense cytoplasmic lysosomes (arrows), secondary lysosomes with heterogeneous electron densities (double arrows), most probably macrophages and many extravasated red blood cells (R). (b) a higher magnification of the (Fig. 21 a). *EM, a X4000, b X6000*.

Table (1): Anova one way statistical analysis for comparison of lipid profile parameters (mg/dl) and Endothelial Nitiric Oxide Synthase enzyme activity (eNOS) (IU/ml) among different studied groups.

Parameter	Negative Control group (Group I)	Positive Control group (Group II)	Simvastatin treated group (Group III)	Atorvastatin treated group (Group IV)	*P* value
Total Cholesterol (mg/dl)	89.67±3.77	90.67 ±1.52 **(A)**	74.83±3.76 **(B)**	72.53±2.98 **(B)(C)**	< 0.01
Triglycerides (mg/dl)	65.33±1.52	67.66±1.22 **(A)**	55.17±1.72 **(B)**	53.37±1.25 **(B)(C)**	< 0.01
HDL (mg/dl)	24±1.93	26.83±1.38 **(A)**	36.50±2.79 **(B)**	40.0±2.16 **(B)(C)**	< 0.01
LDL (mg/dl)	52.60±3.66	50.57±2.58 **(A)**	27.30±5.58 **(B)**	25.33±2.69 **(B)(C)**	< 0.01
VLDL (mg/dl)	13.06±0.30	15.13±0.29 **(A)**	11.03±0.34 **(B)**	11.20±0.97 **(B)(C)**	< 0.01
eNOS (IU/ml)	53.34±12.36	53.51±11.52 **(A)**	85.56±11.57 **(B)**	87.38±11.72 **(B)(C)**	<0.05

N= 7 rats, Sig. P<0.05, non sig. P>0.05
(A): non significant difference as compared to negative control group
(B): significant difference as compared to negative control group
(C): non significant difference as compared to Simvastatin treated group

Apoptosis, or programmed cell death, is an essential physiological process that plays a critical role in development and tissue homeostasis. Apoptosis plays a prominent role in tissue homeostasis; apoptotic signalling can be divided into the intrinsic pathway, which depends mainly on mitochondrial changes, and the extrinsic pathway, which is activated by extracellular signals (Fan *et al.,* 2005).

On the contrary, lung endothelial cell apoptosis is a highly regulated process that is required for normal blood vessel formation and for orderly removal of senescent, injured, and infected cells from tissues. A dysregulated or impaired apoptotic process may,

however, trigger inflammatory responses and contribute to increased morbidity in response to respiratory infectious agents

Wang *et al.,* 2005 stated that the progress of apoptosis is regulated in an orderly way by a series of signal cascades under certain circumstances. The caspase-cascade system plays vital roles in the induction, transduction and amplification of intracellular apoptotic signals. Caspases, closely associated with apoptosis, are aspartate-specific cysteine proteases and members of the interleukin-1β-converting enzyme family. The activation and function of caspases, involved in the delicate caspase-cascade system, are regulated by various kinds of molecules, such as the inhibitor of apoptosis protein, Bcl-2 family proteins, calpain, and Ca^{2+}.

Caspase-3 protein is a member of the cysteine-aspartic acid protease (caspases family). Sequential activation of caspases plays a central role in the execution-phase of cell apoptosis. Caspase-3 is activated in the apoptotic cell both by extrinsic (death ligand) and intrinsic (mitochondrial) pathways. (Salvesen, 2002 and Ghavami *et al.,* 2009).

As this study found that apoptosis was attenuated in the lung cells via a decrease in the caspase-3 expression. These findings help in clarification of mechanisms underlying statins-induced lung toxicity. On the other hand these results suggest a functional relation between phospholipidosis and apoptosis, likely mediated by PLC-g1.

Previous studies showed that Simvastatin has both anti-proliferative and pro-apoptotic effects on vascular smooth muscle cells Simvastatin treatment also causes activation of caspase-3 and significantly decreases the number of pulmonary vascular lesions. Treatment with simvastatin induced apoptosis of endothelial cells in the obliterated vessels and reduced pulmonary hypertension through apoptosis-induced reopening of the occluded vessels, as shown by caspase-3 immunohistochemistry. However, in normal rat lungs, blood vessels were negative for caspase-3 staining (Kaneta *et al.,* 2003 and Kim *et al.,* 2006).

On observing the serum activity of Endothelial Nitric Oxide Synthase Enzyme (eNOS) in Simvastatin and Atorvastatin treated groups, it showed significant increase when compared with the control group.

Upregulation of endothelial nitric oxide synthase (eNOS) was identified as a non lipid lowering mechanism of action of statins in cases of pulmonary hypertension and atherosclosis. They found that Simvastatin and Atorvastatin enhanced the endothelial production of NO, stabilized the endothelial barrier function and promoted anti-inflammatory effects, which lead to atherogenesis (Endres *et al.,* 2000; Bell and Yellon, 2003; Kaneta *et al.,* 2003; Colhoun *et al.,* 2004 and Kim *et al.,* 2006)

The present work showed inhibition of the pulmonary cells apoptosis, as shown from negative casepase-3 expression, although Simvastatin and Atrovastatin administration induced significant increase in the serum activity of eNOS enzyme. In order to clarify and explain these findings, the complexity of NO production and interaction with biological molecules should be highlighted.

Nitric oxide (NO), synthesized from L-arginine by NO synthases, is a small, lipophilic, diffusible, highly reactive molecule with dichotomous regulatory roles in many biological events under physiological and pathological conditions. NO can promote apoptosis (pro-apoptosis) in some cells, whereas it inhibits apoptosis (anti-apoptosis) in other cells depending on which apoptosis mechanism is being activated. This complexity results from a consequence of the rate of NO production and the interaction with biological molecules such as metal ion, thiol, protein tyrosine, and reactive oxygen species. Long-lasting over production of NO acts as a pro-apoptotic modulator, activating caspase family proteases through the release of mitochondrial cytochrome C into cytosol. However, low or physiological concentrations of NO prevent cells from apoptosis. It was found that NO displays antiapototic properties in hepatocytes; human B lymphocytes; endothelial cells; splenocytes; eosinophils; and PC12 cells (Choi, *et al.,* 2002).

The antiapoptotic mechanism of NO is understood on the basis of gene transcription of protective proteins. These include: heat shock protein, hemeoxygenase, or cyclooxygenase-2 and direct inhibition of the apoptotic executive effectors caspase family protease by S-nitrosylation of the cysteine thiol group in their catalytic site in a cell specific way (Sata, *et al.,* 2000). Furthermore, Kotamraju *et al.,* 2001 concluded that NO can effectively inhibit endothelial apoptosis mediated by oxidized LDL. They also stated that antiapoptotic mechanism of NO is linked to its ability to scavenge the lipid peroxyl radicals that are presumably responsible for the apoptotic signaling cascade.

On studying all the previously mentioned data, it was clear that negative apoptotic findings of Simvastatin and Atorvastatin administration in the current study was independent of Caspase-3. Actually, it was relevant to NO production mechanism.

Mattern and Volm, 2004 and Marriott *et al.,* 2006 stated that apoptosis blockade increases the liability of lung tissue to bacterial infection, especially pneumonia and attenuates its defense mechanisms against carcinomas. These findings could explain the

presence of cellular infiltration and thickening of the interalveolar septa in the present study.

5. Conclusion

It was concluded that administration of statins induces toxic effects on the non diseased lung tissues through dependent and non-dependent lipid lowering mechanisms specially, the Nitric Oxide dependant one.

Recommendations

- The current study raises the alarm of using high doses of statins for lipid lowering purposes and highlights the Nitric Oxide dependent mechanisms of their toxicity.
- The use of high doses of statins in a non diseased lung cases should be re evaluated.
- Further in-depth studies of the mechanisms by which statins-induced lung toxicity are recommended, as the new era of research

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