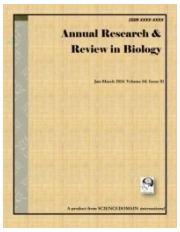

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The Assessment of Tobacco Smoke Toxicity on Selected Tissues from the Cardiovascular and Respiratory Systems of the Albino Rat, an Ultrastructural Study

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Authors' contributions

This work was carried out in collaboration between all authors. They also read, revised and approved the final manuscript.

Original Research Article

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ABSTRACT

Objectives: This work focuses on the effect of cigarette smoke exposure toxicity on the ultrastructure of selected albino rats' cardiovascular and respiratory systems tissues from and their recovery within three months after exposure.
Materials and Methods: Sixty male albino rats (*Rattus norvegicus*) were used in this study. Two groups, each consists of thirty rats. The first group was exposed to the cigarette smoke for three months on a daily basis, using a special modified smoking machine, while the second group (control) was left untreated. The exposure to smoking was followed by a period of three months of non-exposure to smoking as a recovery period. Following each period, the ultrastructural study was performed.

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Results: Cigarette smoke caused ultrastructural changes in the tracheal epithelium, heart ventricles and lung alveoli. Thin section of tracheal epithelium showed low number of cilia, a high degree of cytoplasmic vacuolization. Mitochondria aggregates in the apical portion of epithelial cells, inclusion bodies are present, and disrupted endoplasmic reticulums were also observed. The alveolar epithelium showed damaged multilamellar bodies of type II pneumocyte, together with cytoplasmic vacuolization and chromatin condensation, membrane blebs projecting from the cytoplasm, and degeneration of alveolar epithelium. The ventricular cardiomyocytes revealed mitochondria with deteriorated and partially disrupted or disappeared cristae. Also it showed areas with disrupted Z-discs. After the recovery period, those tissues showed partial recovery.
Conclusion: Smoking induces ultrastructural changes in the respiratory passages and heart that affects the gaseous exchange and may predispose to cancerous changes due to accumulation of toxic and carcinogenic compounds, chromatin condensation and tissue inflammation. Cessation of exposure to cigarette smoking is important in returning most these changes to their normal ultrastructure.

Keywords: Cigarette smoke; Ultrastructural change; cardiovascular system; respiratory system.

1. INTRODUCTION

Smoking is the act of inhaling and exhaling the smoke of tobacco. It is consumed in the form of cigarettes, cigars, chew, pipes or water-pipe [1]. Cigarettes contain more than 4,000 identified chemical compounds including 60 known carcinogens [2]. The gaseous components of mainstream smoke (92% of the total smoke) contain 400-500 different gases which include carbon monoxide, nitrogen oxide, formaldehyde, cyanide, hydrogen and ozone. Particulate matter (8% of mainstream smoke) involves tar product such as pyrene, naphthalene and nitrosamine [2-4] and metal such as cadmium, lead, selenium, mercury, polonium and arsenic [5,6]. According to a report by Public Health Laboratories, Myreland, USA [7], the Jordanian cigarettes contain about twice to third the amount of nicotine and tar which is found in non-Jordanian cigarettes.

Cigarette smoking is linked to 400,000 deaths annually from cardiovascular diseases in the United State alone [7]. There is a clear relationship between the duration and degree of exposure to smoke and incidence of cardiovascular events [8-12]. The deleterious effect of cigarette smoking on the cardiovascular system, would lead to atherosclerosis, coronary artery disease and peripheral vascular disease. Also, smoking has been implicated in the development of aortic dilatation and cerebrovascular diseases [13-15].

The carbon-monoxide and nicotine of tobacco smoking have been mainly implicated in acute cardiovascular disease [16,17]. The nicotine in cigarette stimulates the sympathetic nervous system with consequent effect on the heart rate and peripheral vasoconstriction with consequent elevation of blood pressure [18]. Cigarette smoking increases the risk of developing cancer in different organs of the body and increases their metastasis rates [19,20]. Cigarette smoking is responsible for 90% of all lung cancer, which is the main type of cancer causing death in the world [21-23]. Also, smoking is the major source of chronic obstructive pulmonary disease (COPD), the fourth leading cause of death in the United States [24]. Cigarette smoke contains and generates various reactive oxygen species (ROS) and reactive nitrogen species (RNS), such as superoxide radical, hydrogen peroxide,

hydroxyl radical, and peroxynitrite [25]. The highly reactive radicals can damage the cell membrane and also induce DNA fragmentation [26].

Cigarette smoke produced alteration in the morphology of tracheal epithelium, including mitochondrial swelling and their aggregation in the apical portion of the cells, membrane blebbing, swollen RER and cytoplasmic vacuolation, which are indicators of epithelial injury [27]. Frasca et al. [28] investigated the effect of cigarette smoke on the lung of dogs. A proliferation of alveolar type II cells was observed in the lung parenchyma, almost completely lining some alveoli. In some alveolar type II cells an unusual RER component was observed. Many of the cells showed characteristic lamellar bodies, free ribosomes were more abundant, and the Golgi complex was rarely seen. Occasionally 2-3 vacuoles and what appear to be lipid droplets were seen. Multiple microinvasions of the underlying basal lamina by cytoplasmic processes of these cells frequently occured. In a research done by Lough and other [29], both filtered and unfiltered cigarette smoking showed myocardial mitochondria in irregular size and shape with frequent separation of the outer mitochondrial membrane, increased lipid droplet and enhanced autophagosomal activity. Though, there was no change detected in the myofibers, Pekmez et al. [30] found that the histopathological changes of rat kidney tissue exposed to smoking were partially disappeared after treatment with the antioxidant compound caffeic acid phenethyl ester.

This work focuses on the effect of cigarette smoke exposure toxicity on the ultrastructure of selected albino rats' cardiovascular and respiratory systems tissues from and their recovery within three months after exposure.

2. MATERIALS AND METHODS

Sixty male albino rats (*Rattus norvegicus*), (6-8 weeks old, weighing about 100-150 g) were used in this study. Two groups, each consists of thirty rats; Group I breathed normal clean air, while Group II breathed cigarette smoke only. Group II exposure to smoking was carried out by a single daily dose of one cigarette per rat for a period of ninety days using an electronically controlled smoking machine. The exposure to smoking was followed by a period of three months of non-exposure to smoking as a recovery period. Following each period, rats were anesthetized, dissected and tissues of trachea, lung and heart ventricles were removed for ultrastructural study. At the same time Group I rats were placed in a chamber and were exposed to fresh air for three months and were maintained under standard laboratory conditions including diet, humidity and a temperature of 25ºC. Experimental design was done according to a previous work [31,32,33].

2.1 The Digital Smoking Machine

Exposure of animals to cigarette smoke has been done using an electronically controlled smoking machine [31]. The smoking machine was used to monitor the effects of cigarette tobacco smoke through a vacuum pump. A time controller and an electronic valve controlled the sequence of puffs and fresh air into and out of the inhalation chamber. This sequence was used so that when the fresh air valve opened the smoke control valves closed and vice versa. The design allowed enough intake of tobacco smoke and prevented oxygen deprivation in the inhalation chamber. Each complete cycle of the smoking regimen lasted for 90 seconds and consisted of three successive steps as follows: cigarette smoke was drawn into the inhalation chamber continuously for 30 sec. Then fresh air inlet was opened

for 30 seconds, allowing fresh air to wash out the chamber from the smoke, allowing the rats to inhale fresh air for a period of 30 seconds. This process was repeated seven times.

2.3 Procedure of Transmission Electron Microscopy

2.3.1 Preparation of blocks

Tissues of trachea, lung and heart ventricles were directly cut into tiny pieces, approximately (1 mm3), and then immersed in the Karnovsky´s fixative for 2 hours at room temperature. Tissue specimens were then washed with washing buffer (pH 7.2) for 30 minutes (3 changes), and then post-fixed with 1% osmium tetroxide in distilled water for 1 hours. at room temperature specimens were washed again three times, 10 min each with the washing buffer. Dehydration was done by immersing the tissues for 5 minutes once, in acetone concentrations of 30%, 50%, 70%, 95% and twice in 100% acetone. The specimens were infiltrated in a solution of 50% spurr's medium in acetone for 2 hours, followed by two successive changes of 100% spurr's medium, and left overnight with continuous smooth agitation. The samples were then embedded in pure spurr's medium and left in an oven at 60ºC overnight; to allow full polymerization of the resin [34-36].

2.3.2 Sectioning,stainingandmicroscopy

Before sectioning, each tissue block was trimmed, to expose a suitable area of the tissue section. Silver-gold thin sections were obtained using the ultramicrotome (Reichert-Jung Ultracut E) and a diamond knife, and then mounted on a 200 mesh copper grids. Sections were stained with aqueous uranyl acetate in the dark for 20 min, and washed with boiled distilled water, and then post stained with lead citrate for 10 min. Finally, the stained sections were studied at 60 kilovolts, using Zeiss 10B transmission electron microscope [36].

3. RESULTS

3.1 Studying the Effect of Cigarette Smoke and Its Recovery By Transmission Electron Microscopy

3.2 Effecton the Trachea

Thin sections of trachea from control animals showed normal ciliated pseudostratified columnar epithelium and goblet cell (Figs. 1 and 2). The examined thin section of cigarette smoke-exposed, showed absent to low numbers of cilia, membrane blebbing, a disrupted endoplasmic reticulum, a high degree of cytoplasmic vacuolization in all epithelial cells, and some boundaries between epithelial cells can't be distinguished. Mitochondria aggregates in the apical portion of epithelial cells, inclusion bodies are present, and disrupted endoplasmic reticulums were also observed (Figs. 3, 4, 5 and 6). After the recovery period, tracheal epithelium showed an increase in the number of cilia, at the same time it showed a decrease in the number of blebbing, and in cytoplasmic vacuolization. Also we noticed a disrupted endoplasmic reticulum returning to its normal structure. All these changes indicate a partial recovery in rat tracheal epithelium tissue (Fig. 7).

3.3 Effect on Alveoli of the Lung

Thin sections of lungs from control rats revealed normal morphology of type II pneumocytes (Fig. 8), and type I pneumocytes (Fig. 9). The alveolar epithelium of cigarette smoke-expose drats showed damaged multilamellar bodies of type II pneumocyte, together with cytoplasmic vacuolization and chromatin condensation, membrane blebs projecting from the cytoplasm, pleomorphic mitochondria with partially disrupted cristae, inclusion bodies, and degeneration of alveolar epithelium (Figs. 10, 11, 12, 13 and 14). After the recovery period, thin sections in the alveolar epithelium still showed a condensed chromatin and an increase in the number of pleomorphic mitochondria (Fig. 15).

3.4 Effect on Heart Ventricles

Thin sections of heart ventricles from control rats revealed normal sarcomeres and mitochondria (Figs. 16 and 17). Thin sections of ventricular cardiomyocytes of cigarette smoke-exposed rats revealed pleomorphic mitochondria with deteriorated and partially disrupted or disappeared cristae, and deposited toxic materials. Also it showed areas with disrupted Z-discs (Figs. 18, 19 and 20). After the recovery period, the ventricular tissue of cigarette smoke-exposed rats showed partial recovery of cardiac muscle fibers, and enlarged mitochondria, irregularly arranged with partial disrupted cristae (Figs. 21 and 22).

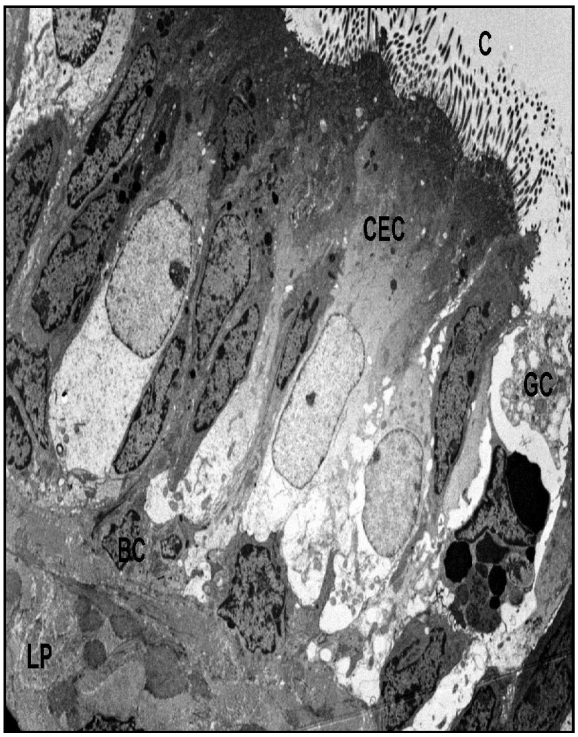

Fig. 1. Thin section of control tracheal tissue. C: cilia, GC: goblet cell, CEC: columnar epithelial cell, BC: basal cell, LP: lamina propria. Magnification: 3000x

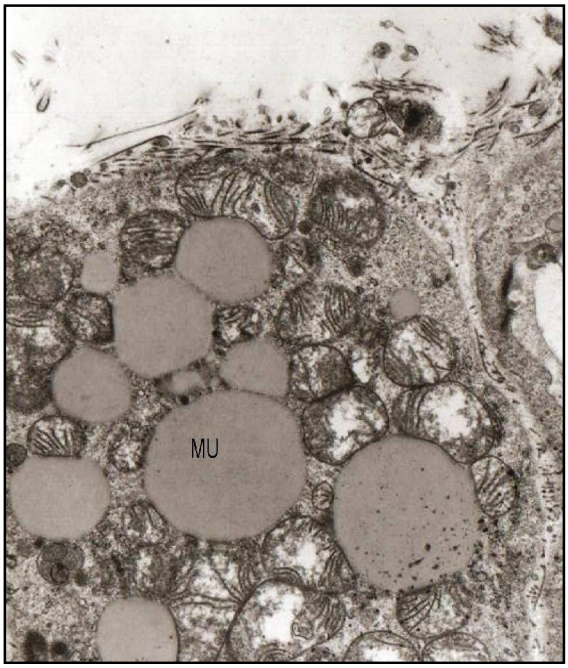

Fig. 2. Closer image at a normal tracheal goblet cell, Mu: mucigen granules. Magnification: 20000 x.

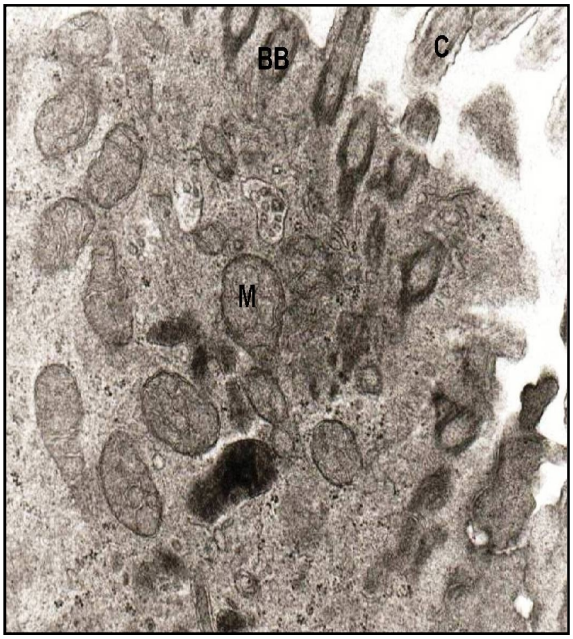

Fig. 3. Thin section in the tracheal epithelium of cigarette smoke-exposed rat, where boundaries between cells can't be clearly distinguished. Mitochondria aggregate in the apical portion of epithelial cells. C: cilia, M: Mitochondria. BB: basal body. Magnification: 62500x

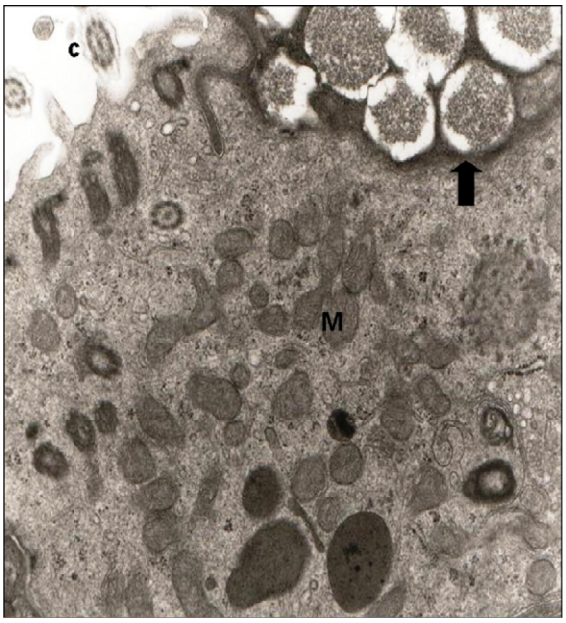

Fig. 4. Thin section in a tracheal epithelium of cigarette smoke-exposed rat, showing low number of cilia. Mitochondria aggregate in the apical portion of epithelial cells. Thick arrow: inclusion body. M: Mitochondrion, C: cilia. Magnification: 40000x

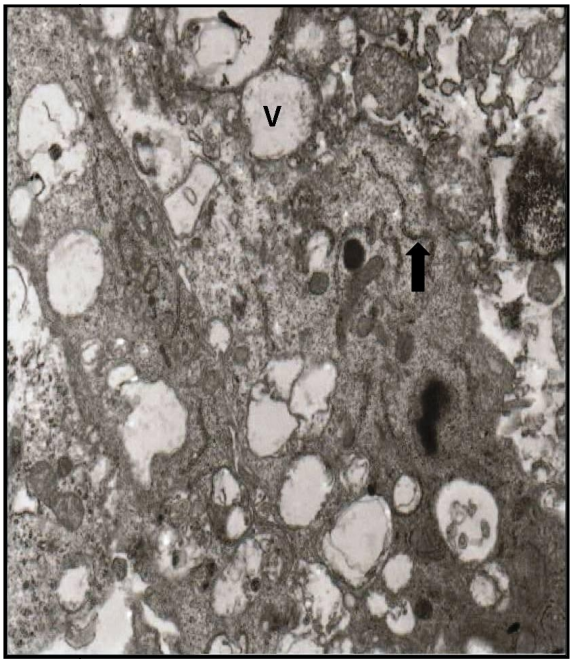

Fig. 5. Thin section in a tracheal epithelium of cigarette smoke-exposed rat, showing a high degree of cytoplasmic vacuolization. Thick arrow: a disrupted endoplasmic reticulum. V: vacuole. Magnification: 20000x

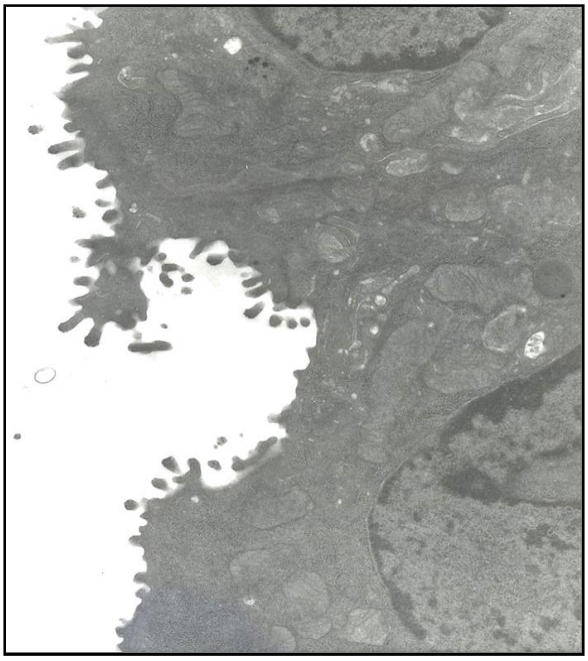

Fig. 6. Thin section in a tracheal epithelium of cigarette smoke-exposed rat. Cilia are totally absent from this field. Total magnification: 12500x

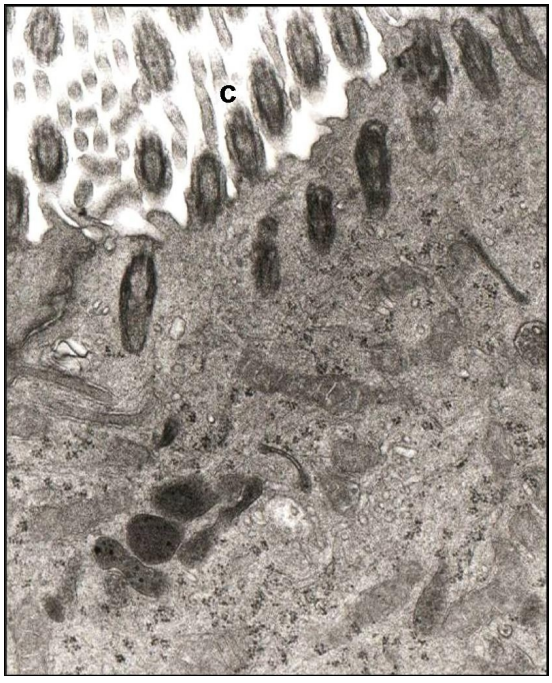

Fig. 7. Thin section in the tracheal epithelium of cigarette smoke-exposed rat after the recovery period. Showing partial recovery of tracheal epithelium. Magnification 50000x

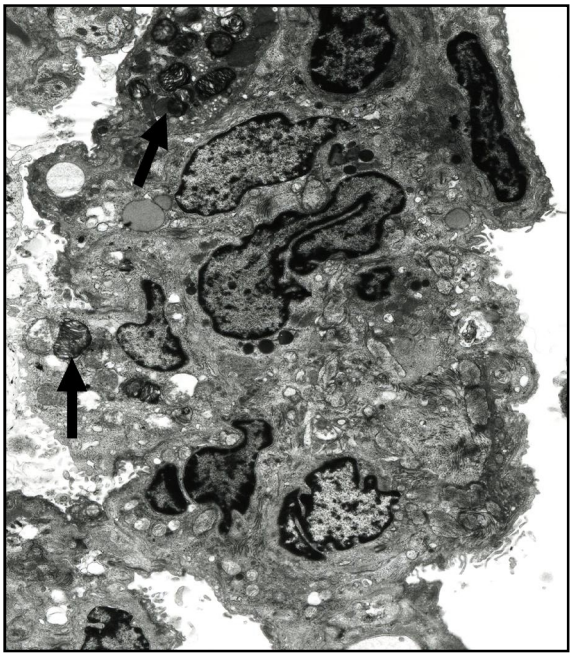

Fig. 8. Thin section of control type II pneumocytes. Thick arrows indicate multilamellar bodies. Magnification: 6356x

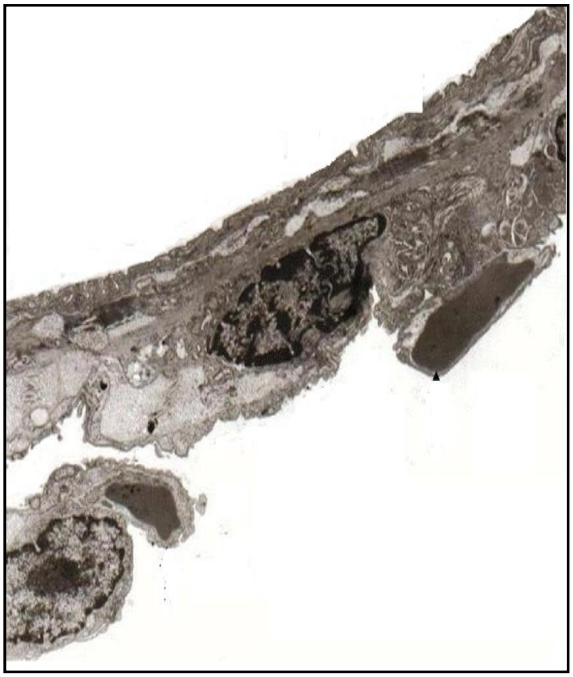

Fig. 9. Thin section of control type I pneumocytes. Magnification: 7874x

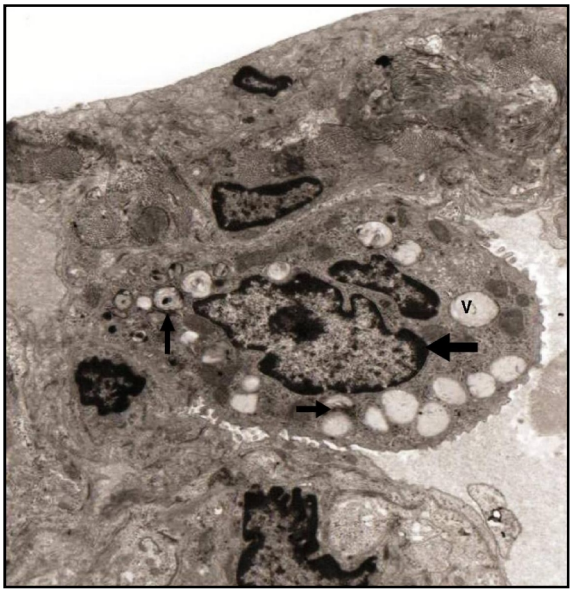

Fig. 10. Photomicrograph from cigarette smoke-exposed rat. Thin arrow: damaged multilamellar bodies. V: cytoplasmic vacuolization. Thick arrow: condensed chromatin. Magnification: 10000x.+

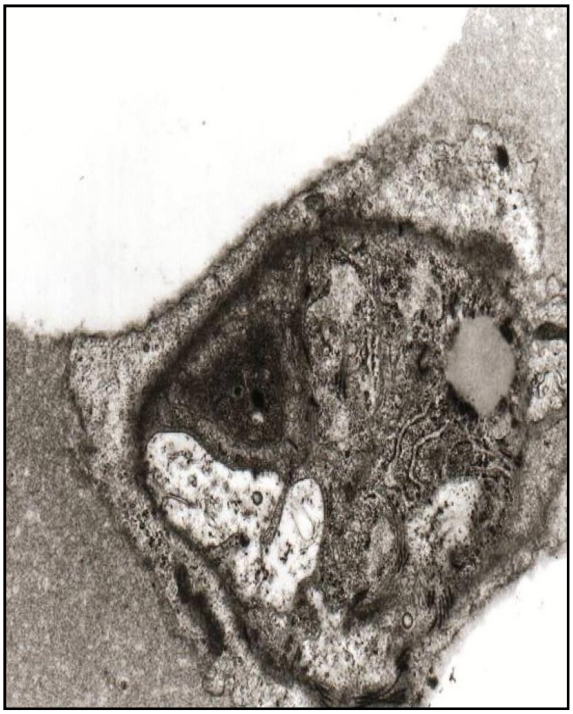

Fig. 11. Photomicrograph from the lung of cigarette smoke-exposed rat, showing degeneration of alveolar epithelium. Magnification: 25000x

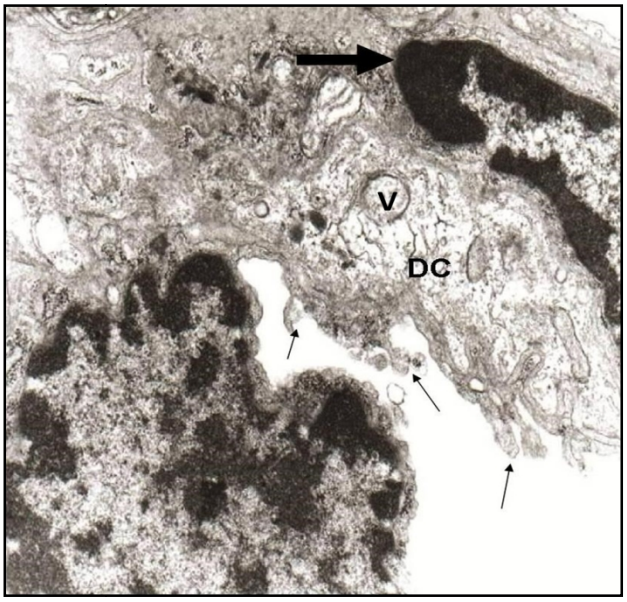

Fig. 12. Thin section in the alveolar epithelium of cigarette smoke-exposed rat, showing condensed chromatin and membrane blebs projecting from the cytoplasm of type II pneumocyte as indicated by thin arrows. DC: area of disrupted cytoplasm. Thick arrow: condensed chromatin. V: vacuole. Magnification: 40000x

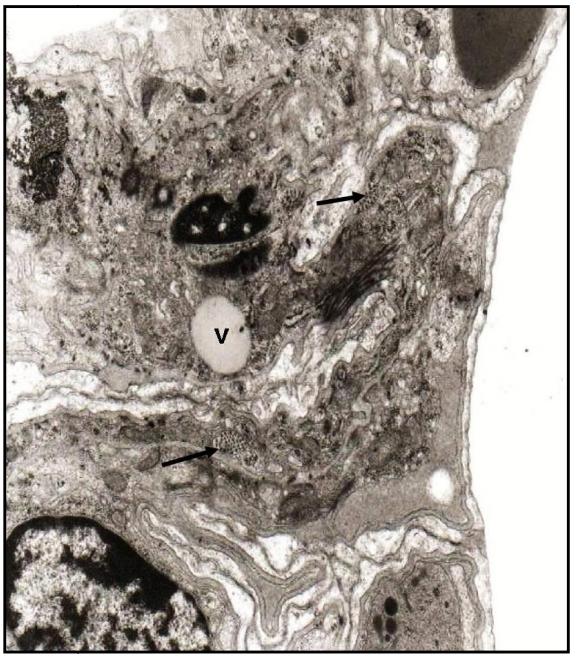

Fig. 13. Thin section in the alveolar epithelium of cigarette smoke-exposed rat, showing condensed chromatin. Thin arrows: inclusion bodies. V: vacuole. Magnification: 25000x

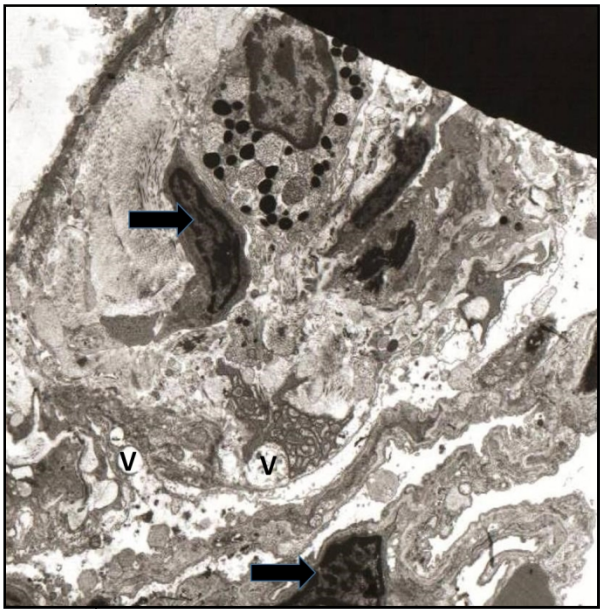

Fig. 14. Thin section in the alveolar epithelium of cigarette smoke-exposed rat, showing condensed chromatin and high degree of cytoplasmic vacuolization. Thick arrow: condensed chromatin V: vacuole. Magnification: 6250x

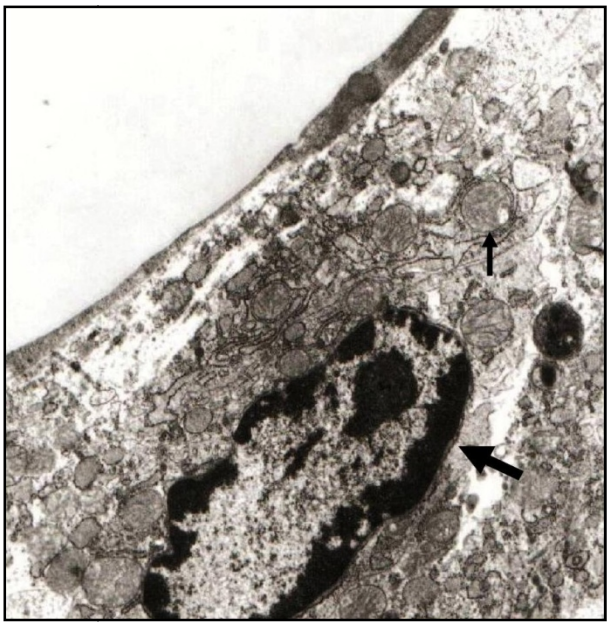

Fig. 15. Thin section in the alveolar epithelium of cigarette smoke-exposed rat after the recovery period, showing condensed chromatin and increase in number of mitochondria. Thick arrow: condensed chromatin, thin arrow indicate mitochondria. Magnification: 25000x

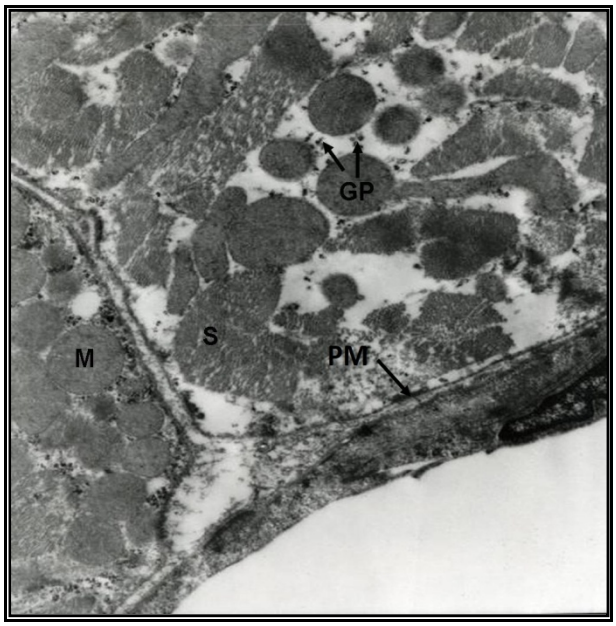

Fig. 16. Closer image of control heart ventricular tissue. GP: glycogen particles, PM: plasma membrane, M: mitochondrion, S: cross-sectional view of sarcomeres. Magnification: 25000x

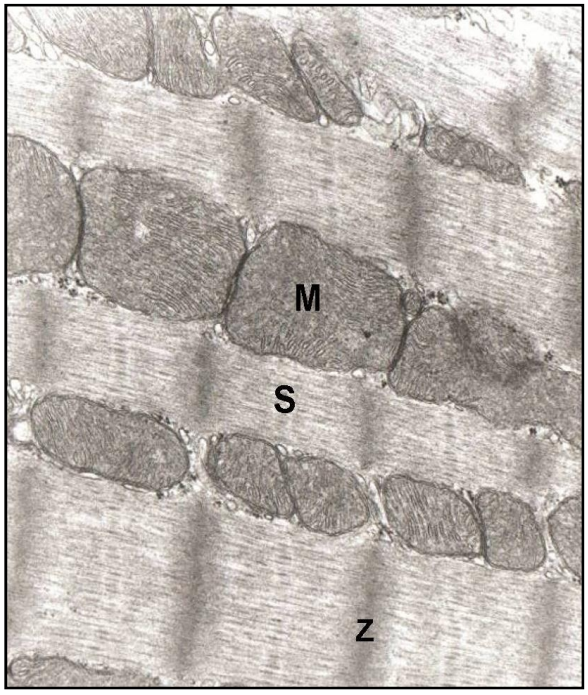

Fig. 17. Thin section of control heart ventricular tissue: M: mitochondrion with normal cristae, S: sarcomeres, Z: Z-line. Magnification: 62500x

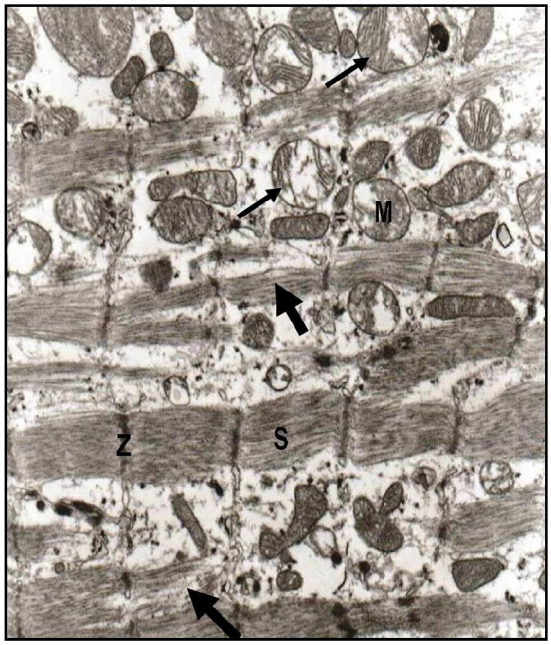

Fig. 18. Thin section of heart ventricular tissue of cigarette smoke-exposed rat. The thick arrows indicate a partial disruption of the myofibrils. Thin arrows indicate pleomorphic mitochondria with partially disrupted cristae. M: mitochondrion, S: sarcomeres, Z: Z-line. Magnification: 5000x

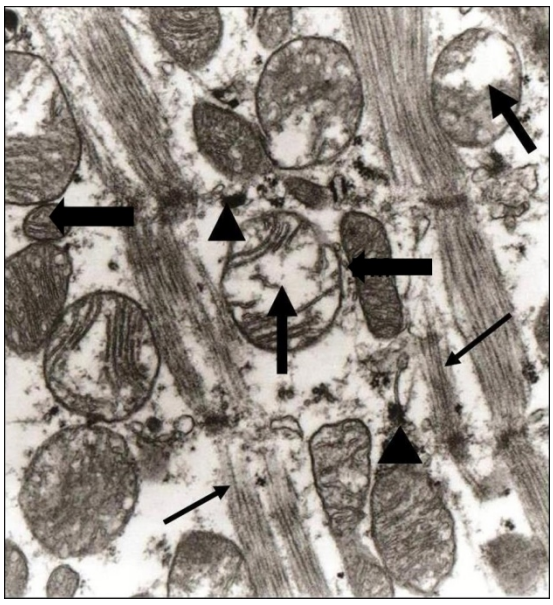

Fig. 19. Thin section of heart ventricular tissue of cigarette smoke-exposed rats. The thin arrows indicate a partial disruption of the myofibrils. Thick arrows indicate pleomorphic mitochondria with partially disrupted cristae. The triangle indicates deposited material. Magnification: 62500x

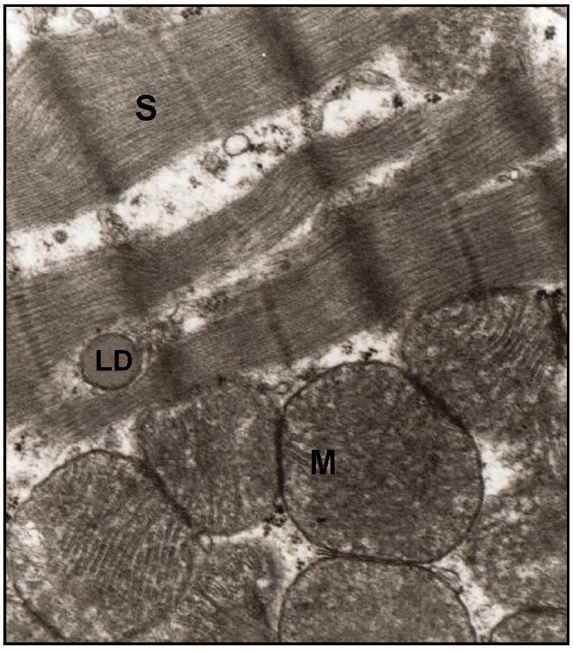

Fig. 20. Thin section of cigarette smoke-exposed rat, showing enlarged mitochondria, irregularly arranged with partial disrupted cristae. M: mitochondrion, S: Sarcomeres, LD: lipid droplet. Magnification: 62500x

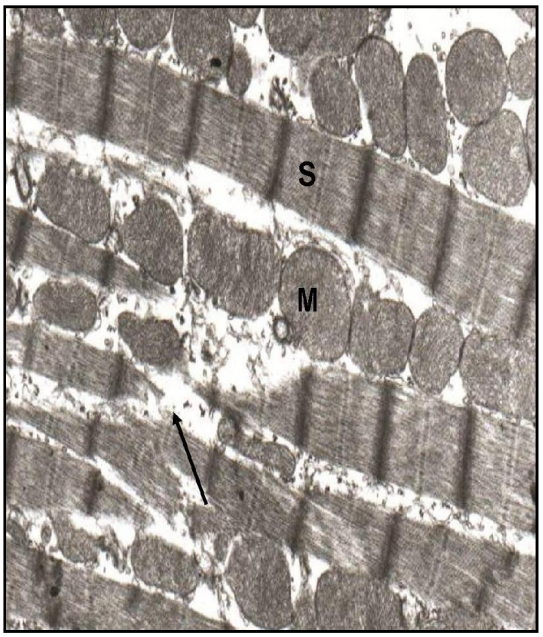

Fig. 21. Thin section of heart ventricular tissue of cigarette smoke-exposed rat after the recovery period. The thick arrows indicate a partial recovery of cardiac muscle fiber. Pleomorphic mitochondria but still partially disruption cristae. M: mitochondrion. S: sarcomeres. Magnification: 25000x

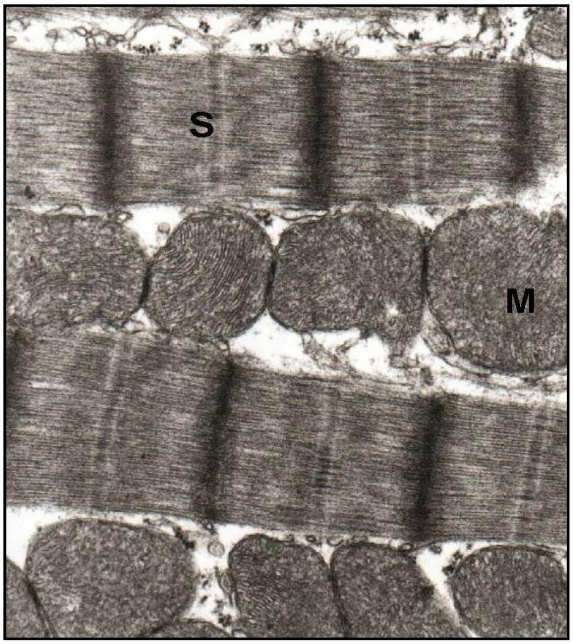

Fig. 22. Thin section of heart ventricular tissue of cigarette smoke-exposed rat after the recovery period, showing partial recovery of cardiac muscle fiber. Enlarged mitochondria with partial disruption cristae. M: mitochondrion, S: sarcomere. Magnification: 62500x

4. DISCUSSION

In our study, mucosa disruption that was frequently observed within the tracheal epithelial cells is due to cell degeneration. Also, ciliary amalgamation that can be viewed as part of epithelial disruption, might result from the hyperplasia of mucus-secreting submucosal glands, and affect the airway clearance mechanisms [37]. Inclusion bodies within the cytoplasm of tracheal epithelial cells were observed as a result aggregate of many poisons, toxicants and carcinogens, like heavy metals especially arsenic and chromium, nitrosamines, nicotine and tar components [27]. The observed loss of cilia on the tracheal epithelium from cigarette smoke-exposed rats may be related to the high degree of nicotine it contains, through its effect on microtubules; polymerization/ depolymerization of tubulin. Acetaldehyde and acrolein are suspected to play a role in the damage of cilia. Acetaldehyde was able to impair the ciliary function and beat frequency, by inhibiting ciliary dynein ATPase activity, and binding to ciliary proteins critical in the functioning of dynein and tubulin, whereas acrolein was found to adversely perturb the cilia by reducing its beat frequency, in cultured bovine bronchial epithelial cells [38]. The examined thin section of cigarette smoke-exposed also showed membrane blebbing, a disrupted endoplasmic reticulum, a high degree of cytoplasmic vacuolization in all epithelial cells, some boundaries between epithelial cells can't be distinguished, mitochondria aggregates in the apical portion of epithelial cells, and disrupted endoplasmic reticulums, which are indicators of cigarette smoke toxicity on tracheal tissue. The effect of cigarette smoke on tracheal tissue of rat showed several morphological changes in the epithelium, including desquamation, loss of cilia and an increase of goblet cells. Activation of serous glands in the submucosa, membrane blebbing, swollen RER, cytoplasmic vacuolation, and cell infiltration were also

noted. These morphological changes were correlated with the amount of toxic substances in the cigarette smoke [37]. The infiltrating inflamatory cells during chronic inflammation, amplifies the tissue damage by releasing more oxygen free radical or through secretion of lytic enzymes [39]. It was found that exposure of rat tracheal tissue explants to varying amounts of cigarette smoke for 10 min, can induce a dose-related blebbing of the apical membrane, and loss of cilia [38].

The alveolar epithelium of cigarette smoke-expose drats showed damaged multilamellar bodies of type II pneumocyte, together with cytoplasmic vacuolization and chromatin condensation, membrane blebs projecting from the cytoplasm, pleomorphic mitochondria with partially disrupted cristae, inclusion bodies, and degeneration of alveolar epithelium. Cigarette smoke causes severe ultrastructural effects especially in lung alveoli due to the differential load of metabolites of cigarette smoke. Al-Awaida et al. [32] showed the cigarette smoking has more severe inhibitory and damaging effects on lung due to the differential load of metabolites of cigarette smoke in correlation with the alterations in enzymes activities. Alarifi et al. [36] studied the ultrastructural changes of pneumocytes of rat exposed to arabian incense (bakhour), Alveolar pneumocytes of exposed animals revealed significant ultrastructural changes which involved the cell organelles and surfactant material of type II cells. Hyperplasia of alveolar cells in the affected lung tissue also observed. In the present study. Neutrophils were recognized infiltrating pulmonary alveoli and accompanied with degenerative and necrotic changes of the alveolar cells. Deposition of collagen fibrils in the alveolar walls was also observed. Hyperplasia of the alveolar epithelium has been described in a variety of experimental and clinical disorders such as pulmonary edema [40] and exposure to chemical irritants [41] carcinogens [42] and radioactive material [43]. The importance of surfactant alterations comes from the important role of surfactant in the prevention of alveolar collapse were observed in the present study, and its act as an active component of the lung host defense mechanism [44,45]. Decreased rate of surfactant secretion can lead to increased density of the surfactant material [46]. It is supposed that some active particulates in the smoke have bound to the cell membrane of type II pneumocytes and affect its capability of surfactant secretion. The pneumocytes hyperplasia is considered as an early response of the alveolar wall to injury [47]. It is suggested that cellular hyperplasia occur through a regenerative process to replace the damaged alveolar cells. Type II pneumocytes are known to be the progenitors of Alveolar type I cells. As in the present study, cigarette smoke caused an accumulation of inflammatory cells in the lung tissue. These inflammatory cells may contribute in damaging of the alveolar and interstitial pulmonary structures through secretion of lytic enzymes and oxygen free radical [39].

In our study thin sections of ventricular cardiomyocytes of cigarette smoke-exposed rats revealed pleomorphic mitochondria with partially disrupted cristae. Also it showed partial disruption of the myofibrils. Lough [29] suggested these changes to be caused by carbon monoxide and that resemble the changes of chronic intermittent hypoxia. In a research done by Lough and other [29], both filtered and unfiltered cigarette smoking showed myocardial mitochondria in irregular size and shape with frequent separation of the outer mitochondrial membrane, increased lipid droplet and enhanced autophagosomal activity. Though, there was no change detected in the myofibers, Moreover, in the present study a mild separation between muscle fibers was observed, this separation will have a negative impact on the capacity of the cardiac muscle of pumping blood efficiently into body organs. The toxic compounds in cigarette smoke may affect muscle fiber through deterioration of both actin-myosine fibers and intercalated disc. The carbon-monoxide and nicotine of tobacco smoking have been mainly implicated in acute cardiovascular disease [16,17]. The nicotine

in cigarette stimulates the sympathetic nervous system with consequent effect on the heart rate and peripheral vasoconstriction with consequent elevation of blood pressure [18].

The toxicity of cigarette smoke in various tissues due to the formation of the radical species. The antioxidant enzyme interacts directly with reactive oxygen species (ROS) to convert them to non-radical products. Overproduction of these radicals by smoking has an inhibitory effect on the enzymes responsible for removal of ROS, which lead to tissue damage.

4.1 Recovery Period

In the present study, tissues of trachea, lung alveoli and heart ventricles showed a partial recovery after cigarette smoke cessation. The findings in the present on reversible effects of smoking support result of Al-Awaida et al. [32], who found that the histopathological changes involved an interstitial inflammation comprised of lymphocytes and plasma cells in lung, portal tract inflammation in liver and mesangial cell proliferation in kidney corpuscles almost diminished in tissues after the recovery period from smoking effects, indicating reversible effects of smoking on tissues and enzymes of the rat. Pekmez et al. [30] found that the histopathological changes of rat kidney tissue exposed to smoking were partially disappeared after treatment with the antioxidant compound caffeic acid phenethyl ester. In vivo studies on the interaction between cigarette smoke and oral peroxidase in smokers and nonsmokers showed that cigarette smoke-induced inactivation of peroxidase activity was in a reversible manner [47]. Cardellach et al. [48] found that chronic smoking is associated with a decrease in enzyme activities of complex IV and III of mitochondrial respiratory chain, which return to normal values after cessation of tobacco smoking. After the recovery period, tissues showed an increasing number of mitochondria, which indicate that high energy, is needed to repair the mechanisms of the cell**.**

One potential limitation in this study was that the post recovery specimens from rats were different from those of post exposure rats, and it would be impossible to take biopsy from the same rat which may cause inflammation in selected tissues. Inspite of this limitation, ultrastructural changes from different rats in the same group yielded the same results.

5. CONCLUSION

Smoking induces ultrastructural changes in the respiratory passages and heart that affects the gaseous exchange and may predispose to cancerous changes due to accumulation of toxic and carcinogenic compounds, chromatin condensation and tissue inflammation. Cessation of exposure to cigarette smoking is important in returning most these changes to their normal ultrastructure.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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