

Structural characterization of rat ventricular tissue exposed to the smoke of two types of waterpipe

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ABSTRACT

Objective(s): this study focused on the effect of waterpipe smoke exposure toxicity on the structure of albino rat's ventricular tissue and their recovery.

Materials and Methods: Albino rats were divided into three groups: control, flavored, and unflavored. The control group was exposed to normal air while the flavored and unflavored groups were exposed to waterpipe smoke for a period of 90 days. Each group was followed by a period of 90 days of fresh air exposure. Following each period, the ventricular tissue was removed for biochemical and histopathological studies.

Results: The ventricular tissues of waterpipe exposed rats showed some degree of separation between cardiac muscle fibers, infiltration of lymphocytes, and congestion of blood vessel. Also, thin cross sections of ventricular cells revealed pleomorphic mitochondria with partially disrupted cristae, partial disruption of the myofibrils, and deposited toxic materials. The unflavored waterpipe has more deleterious effects on heart ventricular tissues than the flavored one. Waterpipe smoke didn't induce apoptosis in the ventricular tissue. We also found very high levels of plasma thiocyanate after exposure to smoke in the flavored and unflavored groups, while the control group showed no increase. After the recovery period, those tissues showed partial recovery.

Conclusion: Waterpipe smoke induces structural changes in the heart ventricle tissues, causing a negative impact on the capacity of the cardiac muscle for pumping blood and may lead to heart attack due to accumulation of free radicals and tissue inflammation. Cessation of smoking is important in returning most of these changes to their normal structure.

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Introduction

Inhaling and exhaling tobacco smoke is referred to smoking. It is consumed in the form of cigarettes, chew, cigars, pipes or waterpipe (1). There are two types of waterpipe tobacco: the unflavored type, called tumbak and the flavored one which could be moassal. The unflavored type is the one that is purely made of moistened shredded tobacco leaves, usually saturated with water before being squeezed, and wrapped in small cube like shape packages to be used in the vessel of the waterpipe (2).

The flavored type tobacco contains honey, molasses, or other syrups, together with glycerol, and other flavoring essences. The flavored, contains approximately (on dry weight bias) 15% tobacco leaves, 47% carbohydrates, 0.53% alcohol, 0.03% nicotine, together with 0.56%, 1.16%, and 1.92% sodium, calcium, and potassium, respectively. It is noteworthy that a pierced layer of tin foil is inserted between tobacco and the charcoal pieces (2, 3).

It has been found that a single waterpipe inhale of a smoking session with either unflavored or flavored tobacco mixture, delivers higher amounts of nicotine, heavy metals such as arsenic (As), lead (Pb), and chromium (Cr), nitrosamines, and both polyaromatic hydrocarbons (PAHs) and carbon monoxide (CO) than the mainstream of cigarette smoke, which are mostly attributable to charcoal releases, than that is delivered by a single cigarette (4, 5).

Cigarette smoking is associated with 400,000 deaths yearly from cardiovascular diseases in the United State alone (6). There is a clear association between the duration and degree of exposure to smoke and the rate of cardiovascular events (7-11). The toxic effect of tobacco smoking on the cardiovascular system, would lead to atherosclerosis, coronary artery disease, and peripheral vascular disease. Moreover, smoking has been implicated in the development of aortic dilatation and cerebrovascular diseases (9, 12-14).

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Among the studies dealing with human narghile smokers from a physiological perspective, it was found that waterpipe smoking, acutely increases coronary risk factors such as heart rate systolic (SBP) and diastolic (DBP) blood pressures and decreases the baroreflex sensitivity (15). Waterpipe smoking has a similar effects on pulmonary function values and respiratory symptoms as deep inspiration cigarette smoking (16). All pulmonary functional tests (PFT) values in smokers were lower compared to the non-smokers, whereas the prevalence and severity of respiratory symptoms (RS) in smokers were higher than non-smokers (17, 18). In addition, in recent studies, increased airway sensitivity to inhaled salbutamol in smokers compared with non-smoking subjects and asthmatics was revealed (16, 19).

The most prominent histological changes in tissues of trachea and lung alveoli of albino rats exposed to the smoke of two types of narghile tobacco were an abnormal proliferation in the epithelium of trachea, disruption of its cilia, and a hyperplasia in the connective tissue of lung alveoli (20). Cigarette smoke caused ultrastructural changes in the heart ventricle cells; the ventricular cardiomyocytes showed mitochondria with deteriorated and partially disrupted or disappeared cristae with areas of disrupted Z-discs (21). Hence, among the scarcity of histopathological research about waterpipe smoking, this research may increase the community concern about the waterpipe use, by revealing the potential adverse effects of two tobacco waterpipe smoke products that differs principally in their components on ventricular cells in animal model (albino rat).

Materials and Methods

The Ethics Committee, in the Science Faculty at the American University of Madaba (AUM), reviewed and approved the use and care of laboratory animals for this study. In this study, sixty male albino rats (*Rattus norvegicus*), (6-8 weeks old, weighing about 100-150 g) were divided into three groups: control (fresh air), flavored (moassal), and unflavored (tumbak). The control group was exposed to normal clean air while the moassal and tumbak groups were exposed to, flavored and unflavored narghile tobacco at electronically controlled dosage. After burning about 20 g of each narghile tobacco type (flavored and unflavored), both flavored and unflavored groups were exposed for a period of 90 days, one session a day. Each exposure session for each group was followed by a 90-day recovery period with fresh air exposure. Following each period, rats were anesthetized, dissected, and the tissues of lung trachea and heart ventricles were removed for biochemical and ultrastructural studies. At the same time the control

group rats were placed in a chamber and were exposed to fresh air for three months under standard laboratory conditions including diet, humidity and a temperature of 25 °C. The experimental design was done according to the previous works (20, 22).

The digital smoke machine

Exposure of animals to narghile smoke has been done using an electronically controlled smoking machine. An inhalation chamber with the dimension of 30 cm in length, 22.5 cm in width, and 10.5 cm in height was made of an 8 mm thick plexiglass, which can host up to five rats (22). The smoking machine was used to monitor the effects of narghile flavored and unflavored tobacco smoke through a vacuum pump. A sequence of puffs and fresh air into and out of the inhalation chamber was controlled by a time regulator and an electronically controlled valve; so that when the fresh air valve was opened the smoke control valves were closed and vice versa. The design allowed enough intake of tobacco smoke and prevented oxygen deprivation in the inhalation chamber. Each complete smoking regimen cycle lasted for 90 sec and consisted of three successive steps as follows: flavored and unflavored tobacco smoke was drawn into the inhalation chamber continuously for 30 sec. Then, the fresh air inlet was opened for 30 sec, to wash out the chamber from the smoke and allowing the rats to inhale fresh air for a period of 30 sec. This synchronized process was automatically repeated 20 times (20 times x 1.5 min exposure = 30 min exposure session) for each group.

Plasma thiocyanate levels of the control and waterpipe smoke groups before and after exposure

Blood samples from experimental and control animals were collected in heparinized tubes. The heparinized blood was centrifuged at 4,000 rpm for 15 min and the thiocyanate levels were determined from the separated plasma. Two ml of the rat plasma was transferred in 15 ml glass-stoppered graduated centrifuge tubes and diluted with water to the 5 ml. Five ml of trichloroacetic acid (20% solution) was added to precipitate the proteins. Five ml of ferric nitrate-nitric acid reagent were added to 5 ml of the filtrate. Absorbance of the test substance was determined at a wavelength of 460 nm. The concentration of thiocyanate in the test sample was calculated by comparing with standard curve (23).

Protocol of light microscopy

Following an overnight recovery period from the last smoke exposure, rats were sacrificed by ether anesthesia and their heart ventricular tissues were gently dissected out, thoroughly washed with normal saline (0.9% NaCl), and then fixed in 10% saline buffered formalin for at least 24 hr. To obtain

Table 1. Effect of waterpipe smoke (flavored and unflavored) on plasma thiocyanate levels in rats

Animal groups	Before exposure $\mu\text{mol/l}$	After exposure $\mu\text{mol/l}$	P-values
Control group (air-exposed rat)	3.41 (3.25-3.59)	3.83 (3.65-4.02)	0.0625
Waterpipe (flavored) smoke exposed rats	3.58 (3.46-3.71)	121 (117-125)	0.0313
Waterpipe (unflavored) smoke exposed rats	3.40 (3.26-3.55)	150 (146-155)	0.0313

Statistical analyses were performed using Wilcoxon signed ranks; a P value < 0.05 was considered significant. Tables shows the medians of the variables (lower-upper interquartile range). The thiocyanate levels were tested for their normality and the kolmogorov tests of normality were significant, indicating a non-normal data

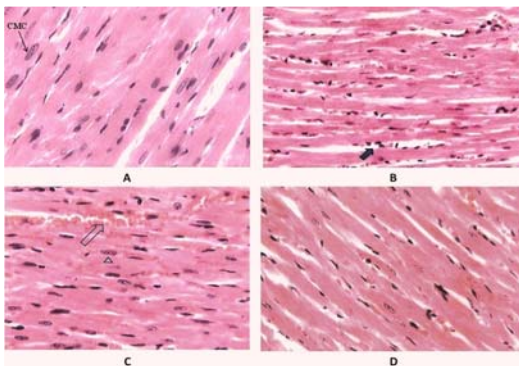


Figure 1. Effect of flavored waterpipe exposed rat on heart ventricular tissue using hematoxylin and eosin staining. A: normal morphology of heart ventricular tissue of air-exposed rat. CMC: cardiac muscle cell. Magnification: 830x. B: heart ventricular tissue of flavored waterpipe exposed rat, showing some degree of separation between cardiac muscle fibers. The arrow indicates lymphocytic infiltration. Magnification: 540x. C: heart ventricular tissue of flavored waterpipe exposed rat. Arrow indicates congested blood vessel. Magnification: 630x. D: Heart ventricular tissue of flavored waterpipe exposed rat after the recovery period. The tissue almost returned to its normal appearance. Magnification: 540x

sufficient fixation, the formalin fixative was used at volume 10 to 20 times more than the volume of tissue pieces. Dehydration was achieved by passing tissues through a graded series of alcohol followed by two changes of xylene. After infiltration in paraffin wax, tissues were embedded in pure paraffin wax (24).

Protocol of transmission electron microscopy Preparation of blocks

Tissues of heart ventricles were directly cut into tiny pieces, approximately (1 mm³), and then immersed in the Karnovsky's fixative for 2 hr at room temperature. Tissue specimens were then washed with washing buffer (pH 7.2) for 30 min (3 changes), and then post-fixed with 1% osmium tetroxide in distilled water for 1 hr at room temperature; specimens were washed again three times (10 min each time) with the washing buffer. Dehydration was done by immersing the tissues for 5 min once, in acetone concentrations of 30%, 50%, 70%, and 95% and twice in 100% acetone. The specimens were infiltrated with a solution of 50% spurr's medium in acetone for 2 hr, followed by two successive changes of 100% spurr's medium, and left overnight with continuous smooth agitation. The

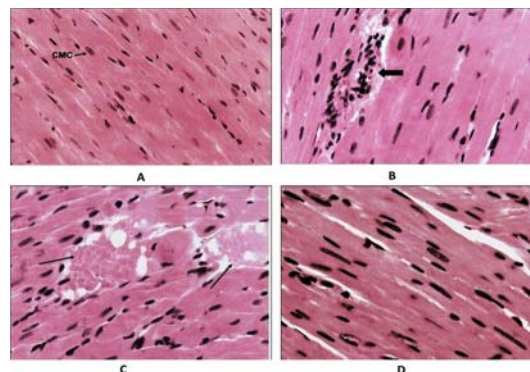


Figure 2. The effect of unflavored waterpipe exposed rat on heart ventricular tissue using hematoxylin and eosin staining. A: normal morphology of heart ventricular tissue of air-exposed rat. CMC: cardiac muscle cell. Magnification: 830x. B: heart ventricular tissue of unflavored waterpipe exposed rat. Arrow indicates lymphocytic infiltration. Magnification: 790x. C: heart ventricular tissue of unflavored waterpipe exposed rat. Arrow indicates congested blood vessel. Magnification: 760x. D: heart ventricular tissue of unflavored waterpipe exposed rat after the recovery period. The tissue almost returned to its normal appearance. Magnification: 1130x

samples were then embedded in pure spurr's medium and left in an oven at 60 °C overnight, to allow full polymerization of the resin (25-27).

Sectioning, staining, and microscopy

Each tissue block was trimmed before sectioning, to expose a suitable area of the tissue section. Silver-gold thin sections were obtained using the ultramicrotome (Reichert-Jung Ultracut E) with a diamond knife and then mounted on a 200 mesh copper grid. Sections were stained with aqueous uranyl acetate in the dark for 20 min, washed with boiled distilled water, and then post stained with lead citrate for 10 min. Finally, the stained sections were studied at 60 kV, using Zeiss 10B transmission electron microscope, Carl Zeiss, Inc, Thornwood, NY (27).

Tracking apoptotic changes in heart ventricular tissue

The presence of apoptotic cell death in heart ventricular tissue was examined using Dead End Colorimetric TUNEL (TdT-mediated dUTP Nick-End Labeling) kit (Promega, USA). Apoptosis was confirmed by electron microscopy, which showed morphological changes on a subcellular level. Electron microscopy is

Table 2. Comparison of histopathological changes in rat ventricular tissue exposed to the smoke of two types of waterpipe and its recovery period

Animal group	Control group (air-exposed rat)		Waterpipe (flavored) smoke exposed rats		Waterpipe (unflavored) smoke exposed rats	
	After exposure	After recovery period	After exposure	After recovery period	After exposure	After recovery period
Lymphocytic infiltration	A	A	P	A	P	A
Congested blood vessels	A	A	P	A	P	A
Pleomorphic mitochondria	A	A	P	P	P	P
Partially disrupted or disappeared cristae	A	A	P	P	P	P
Chromatin condensation	A	A	A	A	P	P
Partial disruption of the Z-disc.	A	A	P	A	P	P
Cardiomyocytes with elongated nucleus	A	A	A	A	P	P
Deposited toxic materials	A	A	P	A	P	P

A: no histopathological changes. P: presence of histopathological

considered to be a valuable, specific and sensitive, and a qualitative methodology for the detection of apoptosis (44).

Statistical analysis

Statistical analyses were performed using Wilcoxon signed ranks; a P -value < 0.05 was considered significant. Table 1 shows the medians of the variables (lower-upper interquartile range). The thiocyanate levels were tested for their normality based on Kolmogorov-Smirnov normality test.

Results

Thiocyanate levels in plasma of waterpipe smoke (flavored and unflavored) exposed rats

The plasma thiocyanate concentration reached high levels of $118 \pm 4.9 \mu\text{mol/l}$ and $147 \pm 5 \mu\text{mol/l}$ in flavored waterpipe and unflavored waterpipe smoke experimental rats, respectively. On the contrary, mean plasma thiocyanate levels of control rats were found to be $3.3 \pm 0.21 \mu\text{mol/l}$ and $3.8 \pm 0.23 \mu\text{mol/l}$, of fresh air exposure, respectively as shown in Table 1. No significant changes in thiocyanate levels were observed for the control group.

Effect of flavored waterpipe exposure on heart ventricular tissue using hematoxylin and eosin staining

Heart ventricular tissue of this group showed some degree of separation between cardiac muscle fibers, congestion of blood vessels, and infiltration of lymphocytes. After the recovery period, the ventricular tissue of flavored waterpipe exposed rats showed partial recovery of cardiac muscle fibers, as shown in Figure 1 and Table 2.

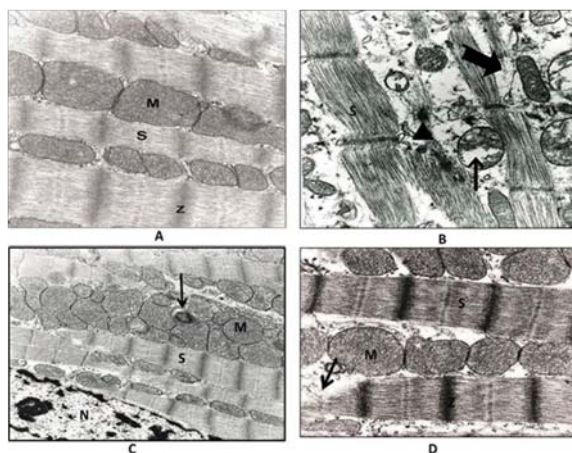


Figure 3. Effect of flavored waterpipe exposed rat on heart ventricular tissue. A: ultrathin section of ventricular cardiomyocytes of air-exposed rat. M: mitochondrion with normal cristae, S: sarcomeres, Z: Z-line. Magnification: 62500x. B: ultrathin section of ventricular cardiomyocytes of flavored waterpipe exposed rat. The thick arrows indicate a partial disruption of the myofibrils. Thin arrow indicates pleomorphic mitochondria with partially disrupted cristae. The triangle indicates deposited material. Magnification: 62500x. C: Ultrathin section of ventricular cardiomyocytes of flavored waterpipe exposed rat, showing enlarged mitochondria, irregularly arranged with partial disrupted cristae. Arrow indicates deposited material, M: mitochondrion, S: sarcomeres, N: nucleus. Magnification: 25000x. D: ultrathin section of ventricular cardiomyocytes of flavored waterpipe exposed rat after the recovery period, showing partial recovery of cardiac muscle fiber. Enlarged mitochondria with partial disruption cristae. Arrow indicates a partial disruption of the myofibrils, M: mitochondrion, S: sarcomere. Magnification: 62500x

Effect of unflavored waterpipe exposure on heart ventricular tissue using hematoxylin and eosin staining

Heart ventricular tissue of this group showed

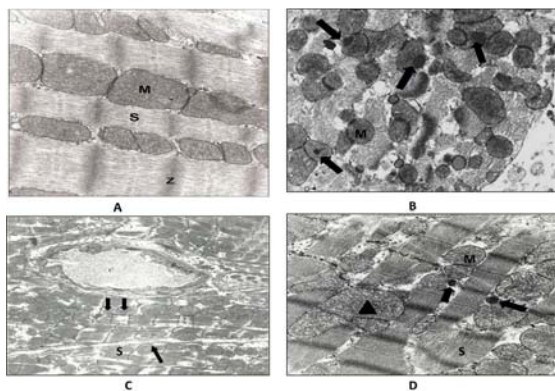


Figure 4. Effect of unflavored waterpipe exposed rat on heart ventricular tissue. A: ultrathin section of ventricular cardiomyocytes of air-exposed rat. M: mitochondrion with normal cristae, S: sarcomeres, Z: Z-line. Magnification:62500x. B: ultrathin section of ventricular cells of unflavored waterpipe exposed rat, showing pleomorphic mitochondria. Arrows indicate deposited material. Magnification 5000x. C: ultrathin section of ventricular cells of flavored waterpipe exposed rat. The two areas enclosed by trapezoidal shapes denote partial disruption of the Z-disc. Arrows indicate partially disrupted mitochondria. C: capillary. Magnification: 5000x. D: ultrathin section of ventricular cells of unflavored waterpipe exposed rat after the recovery period, showing partial recovery of cardiac muscle fiber. Arrows indicate deposited material. The triangle indicates enlarged and pleomorphic mitochondria with partially disrupted cristae. M: mitochondrion, S: sarcomere Magnification: 25000x

lymphocytic infiltration, with some degree of separation between cardiac muscle fibers, congestion of blood vessel, and chromatin condensation. After the recovery period, cardiomyocytes with elongated nucleus and condensed chromatin could still be seen, as shown in Figure 2 and Table 2.

Effect of flavored waterpipe exposure on heart ventricular tissue using transmission electron microscopy

Thin sections of heart ventricles from control rats showed normal sarcomeres and mitochondria. Thin sections of ventricular cells of flavored waterpipe exposed rats showed pleomorphic mitochondria with deteriorated and partially disrupted or disappeared cristae and deposited toxic materials. There were also areas with disrupted Z-discs. After the recovery period, the ventricular tissue of waterpipe exposed rats showed partial recovery of cardiac muscle fibers, as shown in Figure 3 and Table 2.

Effect of unflavored waterpipe exposure on heart ventricular tissue using transmission electron microscopy

Thin sections of heart ventricles from control rats showed normal sarcomeres and mitochondria.

Thin sections of ventricular cells of unflavored waterpipe exposed rats showed pleomorphic mitochondria with partially disrupted cristae and deposited toxic materials. Areas with partial disruption of the Z-disc were also observed. After the recovery

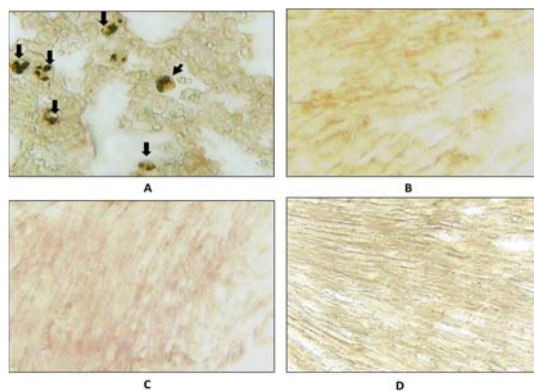


Figure 5. The ability of waterpipe smoke (flavored and unflavored) to induce apoptosis in heart ventricular tissue. A: positive control, with the presence of many dark-brown apoptotic nuclei. Arrows indicate one apoptotic nucleus (TUNEL). B: heart ventricular tissue of air-exposed rat, no dark-brown apoptotic nuclei. C: heart ventricular tissue of flavored waterpipe exposed rat, no dark-brown apoptotic nuclei. D: heart ventricular tissue of unflavored waterpipe exposed rat, no dark-brown apoptotic nuclei

period, the ventricular cells with enlarged and pleomorphic mitochondria with partially disrupted cristae can still be seen, as shown in Figure 4 and Table 2.

The ability of waterpipe smoke (flavored and unflavored) to induce apoptosis in heart ventricular tissue

Waterpipe smoke (flavored and unflavored) didn't induce apoptosis in the heart ventricular tissue of rats after three months of exposure, as shown in Figure 5. We did not detected apoptosis in heart ventricular tissue by electron microscopy techniques.

Discussion

The level of plasma thiocyanate is a good indicator of smoke exposure. Thiocyanate is a metabolite of hydrogen cyanide; amino acids, and proteins in tobacco are the main sources of hydrogen cyanide in waterpipe (28). Distinguishing smokers from non-smokers was determined by measuring thiocyanate levels (23). In this study, no significant changes in thiocyanate levels were observed in the control group. On the other hand, the experimental rats which were exposed to unflavored waterpipe smoke showed more thiocyanate levels than those exposed to flavored waterpipe smoke.

It is well known that cellular antioxidant enzymes, catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx), protect cells and tissues from free radicals and the imbalance between cellular pro-oxidant and antioxidant levels, which may cause oxidative stress and tissue damage. Direct interaction of antioxidant enzyme with radical species converts them to non-radical products. Overproduction of these radicals has an inhibitory

effect on the enzymes responsible for removal of free radicals (29). Ben Saad *et al*, (30) showed that oxidative stress was increased significantly by regular waterpipe smoking. Sharma *et al*, (31) observed the presence of elevated levels of free radicals in peripheral blood neutrophils of waterpipe smokers. Two studies suggested the occurrence of oxidative stress in association with waterpipe smoking, which leads to an imbalance in the production/consumption level of reactive oxygen species (ROS). The first study showed a significant increase in malondialdehyde, as a biomarker for oxidative stress and a significant decrease in vitamin C as a potent antioxidant in waterpipe smokers (32). The second study (33) the potential effect of smoking narghile on oxidation injury was estimated by monitoring the parameters of the (iso) eicosanoid system in narghile smokers. Two *in vivo* biomarkers of oxidative stress (8-epi-prostaglandin F2 alpha (8-epi-PGF2alpha) and malondialdehyde) were increased after a single smoking session. Repeated daily smoking induced a persistent long-lasting oxidation injury.

In this study, the heart ventricular tissues of waterpipe exposed rats showed some degree of separation between cardiac muscle fibers, infiltration of lymphocytes, and congestion of blood vessels. Thin cross sections of ventricular cardiomyocytes of waterpipe exposed rats revealed pleomorphic mitochondria with partially disrupted cristae, partial disruption of the myofibrils, and deposited toxic materials. These morphological changes were correlated with both free radicals and cyanide toxicity which are major components of smoke inhalation, especially in waterpipe smokers (34, 35). Infiltrating inflammatory cells during chronic inflammation, amplifies the tissue damage by releasing more oxygen free radical or through secretion of lytic enzymes (36). These changes may be also caused by carbon monoxide, resembling changes in chronic intermittent hypoxia (37). Moreover, in the present study a mild separation between muscle fibers was observed, which will have a negative impact on the capacity of the cardiac muscle for pumping blood efficiently into body organs (38, 39). Waterpipe smoke (flavored and unflavored) didn't induce apoptosis in the heart ventricular tissue of rats after three months of exposure. Tobacco smoke induced tissue injury, including apoptosis which may be via ROS and nitrogen oxide generation (40, 41)

The toxicity of waterpipe smoke in heart ventricular tissue is due to the formation of the radical species.

In the present study, heart ventricle tissues showed a partial recovery after waterpipe smoke cessation. The detected reversible effects of smoking in this study support the result of Al-Awaida *et al*,

(42) who showed that the histopathological changes such as interstitial inflammation consist of plasma cells and lymphocytes in lung, portal tract inflammation in the liver, and mesangial cell proliferation in kidney corpuscles have been almost diminished in tissues after the recovery period from cigarette smoking effects, indicating recovery effects of smoking on tissues and enzymes of the albino rat. In another study, chronic smoking was associated with a decrease in enzyme activities of complex III and IV of mitochondrial respiratory chain, which returned to normal values after cessation of tobacco smoking (43). After the recovery period, ventricular cells showed an increased number of mitochondria, which indicates that high energy, are needed to repair the mechanisms of the cell.

One potential limitation in this research was that the post recovery specimens were different from those of post exposure, and it would be impossible to take biopsy from the same rat which may cause inflammation in heart tissues. Inspire of this limitation, structural changes from different rats in the same group yielded the identical effects.

Conclusion

Waterpipe smoke induces structural changes in the heart ventricle tissues that have a negative impact on the capacity of the cardiac muscle for pumping blood and may cause heart attack due to accumulation of free radicals and tissue inflammation. Cessation of smoking is important in returning most parts of these changes to their normal structure.

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