ORIGINAL ARTICLE

A Comparative Histological Study of Effects of Shisha and Cigarettes on Trachea of Mice

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ABSTRACT

Objective: To determine the comparative histological effects of Shisha and cigarettes on trachea of experimental animals.
Study Design: It was an analytical experimental randomized control trial.
Place and Duration of Study: The study was carried out in the department of Anatomy, Islamic International Medical College, Rawalpindi, in collaboration with National Institute of Health (NIH), Islamabad. The duration of the study was 6 months.
Materials and Methods: 40 adult male BALB/c mice were used and divided into 3 groups. Control group C was kept in a chamber in fresh air. Group SS was exposed to Shisha smoke and Group CS was exposed to equivalent quantity of nicotine by burning cigarettes. All mice were dissected after 8 weeks and tissues of trachea were examined microscopically and results were compared in experimental groups.
Results: The trachea was examined for three parameters. There was marked mucus cell hyperplasia and submucosal gland hypertrophy in group SS and the difference between group SS and CS was statistically significant ($p<0.005$). The basement membrane in both groups showed marked thickening.
Conclusion: Shisha smoking is not a safe alternative to cigarette smoking. It contains higher level of toxicants in its smoke that cause a much higher disruptions at tissue level as compared to cigarette smoke.

Key Words: *Shisha smoke, Cigarette smoke, Trachea.*

Introduction

"Smoking Kills", "Smoking causes lung cancer", despite these anti tobacco ads on cigarette packs, the use of tobacco products is on the rise. Tobacco is one of the epidemics that is causing most of the deaths in the world and still growing in its consumption across the globe. According to survey by World Health Organization, the expected death toll due to tobacco alone is anticipated higher than the deaths caused by tuberculosis, HIV/AIDS and malaria combined.[1] The survey also revealed that smoking will kill 50% more people than HIV/AIDS in 2015.[2] Even more alarming is that by 2030; death toll will rise to 8 million a year of which more than 80% deaths will be in 3rd world countries.[3] Tobacco is most commonly inhaled in form of a cigarette. A lot of research has been done to highlight the adverse effects of cigarette smoking and it has been found that it is responsible for 90% of lung cancers in men and accounts for 30% deaths due to cancers.[4] Another common form of tobacco intake is through Shisha. This is also known as Water Pipe Tobacco Smoking i.e. smoking through any apparatus that involves passage of tobacco smoke through water before it is being inhaled. The traditional water pipe originated in India in the 15th century and then spread to the Near East countries.[5] Hookahs spread first to Persia and underwent further changes to its original shape to the current known shape.Recently there has been an emergence of the practice among younger adults and adolescents and an estimated 100 million people worldwide smoke Shisha daily. The common perception about this type of smoking of being less harmful than cigarette smoking is leading to tolerance of this practice.[6] A widespread perception among smokers is that the water, through which the smoke bubbles, filters the toxic components, rendering the smoke less harmful than cigarette smoke. On the contrary a single water pipe smoking session yields 20 times the amount of polycyclic aromatic hydrocarbons, formaldehyde, acetaldehyde and acrolein found in mainstream cigarettes; all of these are the prime cause of cancer.[7] It contains the addictive drug nicotine and is as toxic as cigarette smoke. Moreover depending upon the frequency of puffing, depth of inhalation, and length of the smoking session, hookah smokers may absorb higher concentrations of the toxins as compared to cigarette smoke.[8]

Cigarette smokers take 8 to 12 puffs over 5-7

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minutes, inhaling a total of 500-600ml of smoke. In contrast, water pipe sessions typically last 30-60 minutes, during which the smoker may take 50-200 puffs inhaling 500ml of smoke in each puff. Thus a single session produces 50,000ml of smoke.[9,7] There is very little research done addressing the tobacco effects of water pipe smoking despite the fact that there are millions of current water pipe smokers especially the youth and that water pipe smoking is spreading globally. This research was done with an aim to compare the histological effects of Shisha and cigarette smoke inhalation on trachea among study groups.

Materials and Methods

This study was an analytical experimental randomized control trial and was approved by the Institutional Review Committee of Riphah International University before its commencement. Forty adult male BALB/c mice having weight of 35-45g and age between 10-12 weeks were obtained from animal house of NIH, Islamabad where they were kept under standard laboratory conditions. All mice were acclimatized for one week. These mice were maintained on pelleted diet which was prepared in the animal house. They were kept at 12 hours light and dark cycle in a room at 22-24 °C and were given food and water ad libitum.

Animals were randomly divided into 3 groups. The control group C had ten mice that were kept in a chamber exposed to fresh air. Two experimental groups with fifteen mice each were designated as group SS that received Shisha smoke and group CS that was exposed to cigarette smoke. Whole body inhalation exposure was given using a locally made plastic chamber designed according to the specifications of World Health Organization.[10,11] The water pipe apparatus consists of a head to hold 10 to 20g of tobacco. It was connected to a body below which was a bowl half filled with water. A tube was connected to the head that passed through the body and was submerged in water in the bowl. A hose and a mouthpiece were connected to the bowl above the level of the water. 10g of Shisha flavor was placed in the trough on top and covered with aluminum foil with small holes in it. A piece of hot coal biscuit was placed over the foil. The smoke was sucked by a manual vacuum pump through the mouthpiece, which drew air over the burning charcoal and through the tobacco creating an aerosol consisting of volatilized and pyrolized tobacco components (Fig-Ia). The smoke then bubbled into water jar and a post bubbling mainstream smoke was carried via a connecting pipe into the smoke inhalation chamber of experimental group SS. Mice were exposed to Shisha smoke in morning and evening, one puff /2 sec, 5days/week for 8 weeks. Shisha flavor contains approximately 2.5mg of nicotine.[12] The smoke-exposure group CS was exposed to nicotine concentrations equivalent to Shisha smoke group. Nicotine content /cigarette in side stream smoke[8] is 0.12mg. So, nicotine content in 20 cigarettes was 2.4mg. Cigarette smoke was given in a chamber of same dimension as Shisha smoke inhalation chamber. 20 commercial non filtered cigarettes were placed vertically in a plastic stand with holes and were ignited with a lighter (fig-Ib). Mice were exposed to cigarette smoke in morning and evening, 5 day/week for 8 weeks. The mice in Shisha and smoke groups were exposed to 5 min of smoke followed by 5 min of air until all Shisha flavor and cigarettes were consumed, which took 1-1$^{1}/_{2}$ hours. All the animals were sacrificed at the end of 8th week. They were dissected and trachea was removed and preserved in containers containing 10% formalin. Tissue processing and embedding was done in paraffin. Slides were prepared and stained with haematoxylin and eosin. Special staining was done with Periodic acid Schiff to demonstrate thickness of basement membrane and mucus cells in tracheal epithelium. Microscopic study was done under 40X objective of a CX 21 light microscope. Slides were studied for submucosal gland hypertrophy by calculating the Reid's Index. Mucus cell hyperplasia was determined by counting the number of mucus cells/unit area and thickness of basement membrane was also measured. All measurements were taken by using an ocular micrometer fitted into the eyepiece of the microscope. Statistical analysis was done in SPSS version 20.0. Results were compared by applying t-test and ANOVA. A p–value of < 0.05 was considered as statistically significant.

Results

Mucus cell hyperplasia was observed qualitatively as well as quantitatively. Special staining with PAS was done to count the number of mucus cell/unit area. 66.7% of mice in group CS showed mucus cell

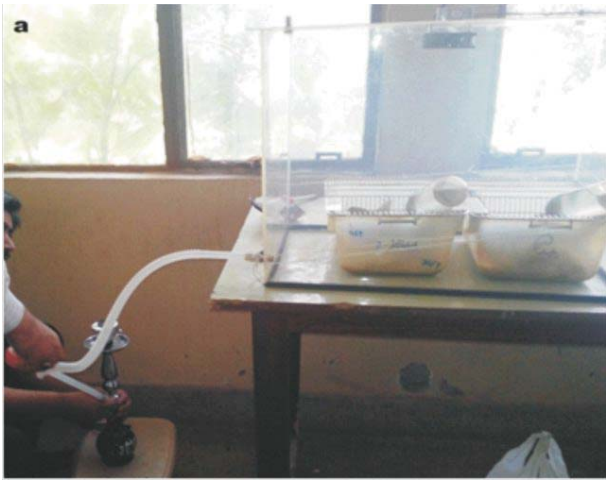

Fig 1: Photograph showing generation of Shisha smoke with a manual vacuum pump (a) and a whole body cigarette smoke exposure chamber showing burnt cigarettes (b).

hyperplasia while it was seen in 100% mice in group SS. The number of PAS positive mucus cells/unit area were counted and compared with the control. The average number of mucus cells in control group was 3±0.94 whereas it was 4.87±1.50 in group CS and 6.67±1.39 in group SS (Table I). The experimental groups showed mucus cell hyperplasia with respect to the control group whereas the hyperplasia was more marked in control group SS (Fig 4) as compared to control group CS with a p-value of 0.002 (Table I). This shows a statistically significant difference among smoke exposure groups. Hypertrophy of submucosal glands was observed in experimental group and was confirmed by taking gland to wall thickness ratio (Reid's Index). Control group showed normal distribution of submucosal glands (Fig 3a). In the smoke exposure groups normal ratio of mucus to serous cells was disturbed with more number of mucus cells in the acinus. Group SS showed marked submucosal gland hypertrophy as compared to Group CS with Reid's index more than 0.4 (Fig 3 b&c). In experimental group CS, gland hypertrophy was present in 8 (53.3%) mice and in 7 (53.3%) mice it is absent. In experimental group SS, gland hypertrophy was present in 14 (93.3%) mice and absent in 1 (6.7%) mice (Fig 2). The difference was statistically significant (p-0.035). Average thickness of basement membrane of trachea was 3.87µm±0.969 in control group, 6.81µm ±1.813 in group CS and 6.67µm±1.948 in experimental group SS (Fig 4). Groups were significantly different from each other ($p<0.001$) (Table I). Control group had significantly lower average thickness of basement membrane of trachea as compared to experimental group CS ($p<0.001$) and experimental group SS (p-0.001) while the difference between group CS and group SS was insignificant ($p = 0.964$) (Table I).

Discussion

The deleterious effects of tobacco on various body organs are well known. A lot of research is still going on to unveil these effects and to curb the tobacco epidemic. The growing awareness of the adverse effects of cigarette, which is the commonest form of tobacco intake, has unfortunately urged people to look for alternate ways to fulfill their craving for nicotine. This resulted in the reemergence of the

Table I: Post hoc comparison of number of mucus cell/unit area and thickness of basement membrane of trachea in µm (n=40)

Parameters	Mucus cell hyperplasia		Thickness of basement membrane	
	Mean Difference	p-value	Mean Difference	p-value
Control group vs. Group CS	-1.86	0.005	-2.94	0.001
Control group vs. Group SS	-1.67	< 0.001	-2.78	0.001
Group CS vs. Group SS	-1.80	0.002	-0.16	0.964

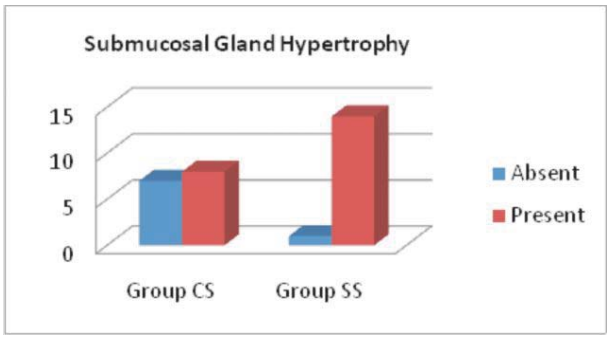

Fig 2: Graph showing submucosal gland hypertrophy in group CS and SS

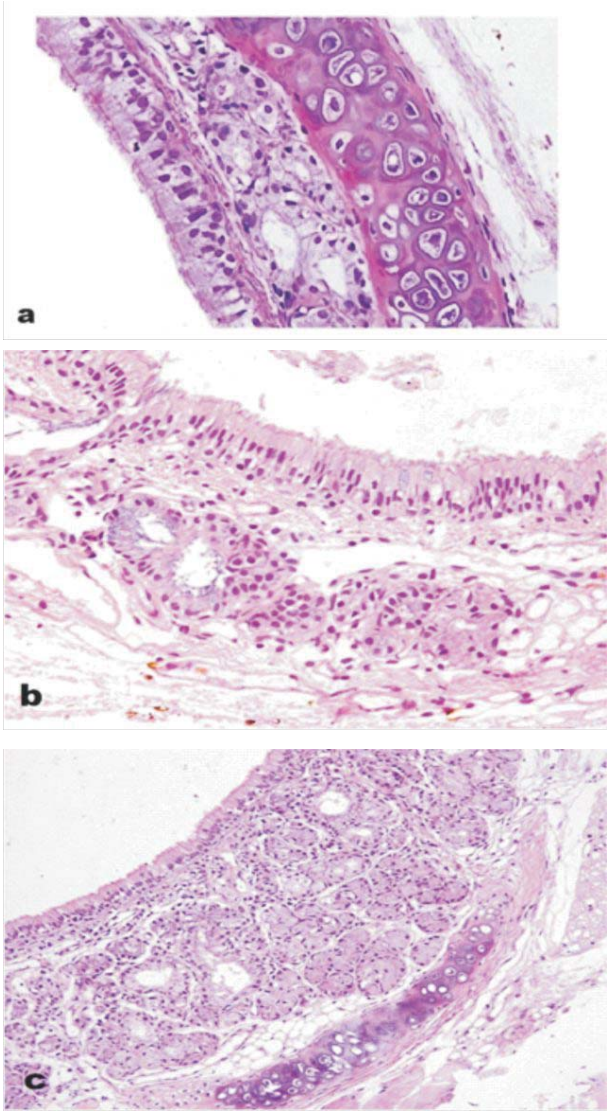

Fig 3: Photomicrographs of trachea of group C showing normal trachea (a), group CS showing mild submucosal gland hypertrophy (b) and group SS showing marked submucosal gland hypertrophy (c). H& E stain 40X

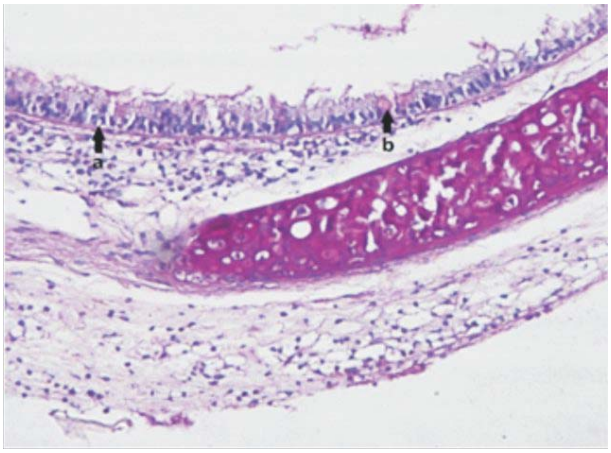

Fig 4: Photomicrograph of trachea of group SS showing thickened basement membrane (a) and PAS positive mucus cells (b). PAS stain. 40X

centuries old practice of water pipe smoking commonly known as "Shisha". The misconception that the water filters the smoke has led to a wide acceptance of this practice that has resulted in a mushroom growth of Shisha cafés all around the world. Quantitative differences were seen in the trachea among experimental groups in terms of mucus cell hyperplasia, submucosal gland hypertrophy and thickened basement membrane. All these pathological tissue changes are interconnected and occur as a consequence of disruption of the normal mucocilary clearance (MCC).[13] MCC is a self clearing mechanism of the airway to remove inhaled pathogens and particulates and forms an important component of lung innate immunity. Effective mucus clearance is essential for lung health, and airway disease is a consequence of poor clearance. Inhaled tobacco smoke contains toxic chemicals out of which nicotine, acetaldehyde[14] and acrolein[15] have been identified as the main culprits that negatively affect MCC by increasing mucus secretion and decreasing ciliated cell numbers. In this research the experimental groups CS and SS showed marked mucus cell hyperplasia with respect to the control group C. Cigarette smoke induced airway mucus hyper secretion has been described previously [16,17] and is attributed as one of the major characteristic of airway remodeling in COPD.[14] The findings in group SS are consistent with two previous studies by Al Esawi[18] and Shraideh[19] on rodents exposed to water pipe tobacco smoke. In this current research, Shisha

group showed greater degree of hyperplasia than cigarette group which is a new finding as no comparative histological study has been done yet. This can be explained on account of higher concentrations of acrolein and acetaldehyde in water pipe tobacco flavor even when its nicotine content was equal to cigarettes. This has been reported by Shihadeh[12] and Chaouachi.[8] According to them higher retention of aldehydes especially acrolein, which is a powerful mucus secretagogue, is seen in the respiratory tract of water pipe smokers as compared to cigarette smokers. Another feature in abnormal MCC is the submucosal gland hypertrophy showed by the Reid's Index.[20,21] This method was introduced by Reid in1960 as a tool to measure severity of chronic bronchitis. It is a proportion of gland thickness to bronchial wall thickness and has the advantage that all glands of a section of trachea or bronchus are covered and results are not influenced by wrinkling of the bronchial mucosa. The results of submucosal hypertrophy in experimental groups were significantly different from the control. The number of acidic glycoprotein containing secretary cells was increased while neutral glycoprotein containing secretary cells were decreased. This finding is consistent with finding by Dye[14] and Shraideh.[22] According to them prolonged submucosal inflammation induced by toxicant injury by cigarette smoke causes hypertrophy of submucosal glands with a shift to acidic, sialidase resistant intracellular glycoproteins. Similar results have been reported by Carter[23], Ying Le,[24] Stefano[17] and Soltani.[25] Presence of glandular hypertrophy in this research in group SS is consistent with findings of a study by Shraideh.[19] Higher degree of hypertrophy in group SS can be explained on account of greater oxidative stress induced by Shisha smoke as compared to cigarette smoke as indicated by Chaouachi[8], though no comparative histological study is done to support this finding. Thickness of basement membrane was measured in Periodic Acid (PAS) stained slides. The results were significant with respect to the control group C. Both experimental groups CS and SS showed an increased thickness of basement membrane. These findings are in agreement with earlier findings by Fischer[26] , Soltani[25] , Carter[27] , and Chung.[28] According to Chung, basement membrane thickening in trachea is attributed to cigarette smoke induced extracellular matrix changes that manifest in the form of deposition of type I and III collagen fibers.

In his research, Shields[29] observed that the basement membrane in tracheal epithelium was thickened in rats exposed to cigarette smoke for two weeks. He explains to it to be due to augmented height of the hypertrophied epithelial cells. Soltani[25] in his review on airway remodeling in Chronic obstructive pulmonary disease (COPD) explains that immediately below the true basement membrane (basal lamina) of the epithelium is the reticular basement membrane (Rbm) or lamina reticularis that forms a loose mesh beneath the true BM in normal subjects. Eosinophilic inflammation leads to thickening of the Rbm in COPD induced by cigarette smoke. In this study the basement membrane was observed under light microscope using 40X objective. At this magnification the parts of basement membrane could not be seen separately. The thickening of basement membrane can either be due to deposition of collagen in the basal lamina or in the Rbm. Chung and Soltani are of the same view that it is the thickening of Rbm that leads to thickening of the basement membrane in trachea of COPD patients. The results are consistent with comparative studies done on levels of toxicants in Shisha cigarette smoke which all showed a much higher levels of carbon monoxide (CO), nicotine, para-amino hydrocarbons (PAH), reactive oxygen species (ROS), acrolein and aldehydes in Shisha smoke. The results of this research rejected the null hypothesis thereby making the alternate hypothesis true that states that Shisha is more harmful than cigarette.

Conclusion

This research provided statistically significant evidence that Shisha smoking is more harmful than cigarette smoking. The comparative histological features showed that Shisha smoke produced greater degree of tissue damage as compared to cigarette. This will help negate the belief of Shisha being a safer alternative to cigarette.

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