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RESVERATROL ATTENUATES HISTOLOGICAL ALTERATIONS IN TRACHEA OF MICE INDUCED BY SECONDHAND SUBCHRONIC EXPOSURE OF CIGARETTE AND WATERPIPE TOBACCO SMOKING

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Abstract

Tobacco smoking is considered the primary causative agent for lung cancer and chronic obstructive pulmonary disease. Their particulate materials and gaseous phase contain multiple toxic chemicals and free radicals that induce oxidative stress in mammalians. Many therapeutic drugs and natural herbs were tested in the last 50 years to examine the possibility of these drugs to have prophylactic effects against the cytotoxicity of tobacco smoking. Resveratrol (RSV) is a promising exogenous antioxidant that gives raised in the last 20 years due to its high free radical scavenging capacity and ability to act as an antioxidant, anti-inflammatory and anti-aging agent against many toxins and heavy metals.In this study, it was proposed whether the antioxidant RSV has an ameliorative effect against the cytotoxicity of two wild forms of tobacco smoking: cigarette (CS) and waterpipe (WPS) tobacco smoking,on the trachea of experimental mice. Forty-eight adult male albino BALB/c mice (6-8 weeks old, 22 ± 3 grams body weight) were randomly assigned into six treatment groups; each group contains eight mice. RSV was intraperitoneally injected in mice (25 mg/kg/day). Mice were exposed to smoking using a modified smoking machine. The experimental duration was six consecutive weeks. Mice were euthanized by physical cervical dislocation for dissection and obtaining the trachea. Tracheasamples were sectionedat 5 µm thickness using a rotary microtome, stained with hematoxylin and eosin,to observe the histopathological characteristicsin trachea using light microscopy. The results of light microscopy of the trachea in this study showed that RSV reduces the amalgamation of cilia, reduces the hypertrophy of submucosal glands and has an anti-inflammatory effect against cigarette and waterpipe tobacco smoking. In conclusion, RSV exhibits a partial ameliorative effect against the cytotoxicity of both cigarette and waterpipe tobacco smoking; it was not able to completely protect the trachea against the above-mentioned inhaled toxins.

Key words: Resveratrol, Microscopy, Trachea, Tobacco smoking.

Introduction

World Health Organization (WHO) reported in 2017 that tobacco smoking causes 10% of death cases among humans. Unfortunately, only 1% of all global funding of health programmes are spent on monitoring and preventing the spread of smoking habits(WHO, 2017; Drope *et al*., 2018). Cigarette tobacco smoking is associated with cellular oxidative damage and could increase the risk of cancer in various organs in human and animal studies (Khani *et al*., 2018). In the last three decades, waterpipe smoking and all its shapes and names like (narghile, hookah and shisha) have been widely spreading around the world especially in middle eastern countries (Salloum *et al*., 2019). The vapor of waterpipe tobacco smoking contains toxicants include nicotine, tar, carbon monoxide, volatile organic compounds, heavy metals, polycyclic aromatic hydrocarbons and heterocyclic aromatic amines, which have been reported to increase free radicals formation and induce oxidative stress (Shihadeh *et al*., 2015; Al-Awaida *et al*., 2015).

Reactive molecules "free radicals" can damage tissues by reacting with critical sulfhydryl bonds in proteins, nucleotides in deoxyribonucleic acid (DNA) and polyunsaturated fatty acids in cellular membranes. Tobacco smoke induces oxidative stress in mammals through increasing level of reactive oxygen species (ROS) and reactive nitrogen species (RNS) that generate free

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radicals that may develop to initiate tumor formation that could evolve into cancer (Dreher and Junod, 1996). Chronic exposure to tobacco smoke containing irritant chemicals could induce chronic obstructive pulmonary disease (COPD) (Yoshida and Tuder, 2007).

Antioxidants are chemicals able to inhibit or suppress the action of free radicals, therefore these chemicals could lower the risk of oxidative stress and preserve the biomolecules of the cells from modifications and damages. Resveratrol (RSV) is 3,5,4' trihydroxystilbene with molecular formula $C_{14}H_{12}O_3$ and molecular weight 228.24 g/mol. It is classified as an exogenous antioxidant compound extracted from plants espacillay from red grapes and has numerous beneficial chemophysiological activities in mammalian organisms (Pandey and Rizvi, 2009; Perrone *et al*., 2017). Morever, RSV is well known as anti-oxidant, anti-inflammatory, anti-aging and anti-carcinogenic compound with vast number of benefits and applications in research, treatments and clinical trials in medical fields (Jang *et al*., 1997; De La Lastra and Villegas, 2005; Das and Das, 2007; Athar *et al*., 2009).

This article aims at finding out the ameliorative effect of resveratrol against the cytotoxicity of cigarette and waterpipe tobacco smoking by investigating the histopathological alternations in trachea using light microscopy technique.

Materials and Methods

Resveratrol (RSV)

Trans-RSV was purchased from Sigma-Aldrich (USA). The purity of RSV was 99.65% HPLC. The daily dose of RSV was (25 mg/kg/day) according to Kandil *et al.,* (2017). Phosphate buffer saline (PBS), (0.1 M, pH 7.2) was used as vehicle during preparation of working solution.

The Cigarettes

Red labeled (L&M) mark cigarettes was purchased from available local markets (Philip Morris, Jordan). The packet cover showed a health caution certified by the Ministry of Health in Jordan. Packet cover listed magnitude of some ingredient in each cigarette (10 mg tar, 0.8 mg nicotine and 10 mg CO).

Tobacco honeyed-maassal and waterpipe preparation

Honeyed-Maassal was purchased from available local markets (Two Apples Flavoured Molasses, Mazaya, Jordan). The packet cover showed a health caution certified by the Ministry of Health in Jordan. Packet cover did not illustrate the concentrations of all its ingredients (Tobacco, Molasses, Glycerine, Flavours and 0.05% nicotine). Five grams of maassal was uploaded to waterpipe head and the vase was half filled with tape water. For burning tobacco, one fast lighting disk 40 mm charoal was burned upon a perforated aliminium foil covering the waterpipe head.

Experimental animals

This study was operated according to instructions of the scientific committee in the School of Science at the University of Jordan in terms of animal handling and care. These instructions are consistent with the NIH guide for the care and use of laboratory animals(National Research Council, 2011). Male albino BALB/c mice were chosen as animal model to perform this study. Mice were purchased from (Animal household / The University of Jordan). All adult males were aged of about eight weeks, weight 22 ± 3 grams. Forty-eight mice were divided randomly into 6 experimental groups; each group contains 8 mice. Group (Control): Mice were exposed to fresh air only. Group (RSV): Mice injected with 0.2 ml of RSV, interperitoneally (IP). Group (CS): Mice were exposed to cigarette smoking. Group (CS+RSV): Mice were exposed to CS and 0.2 ml RSV, (IP) injection. Group (WPS): Mice were exposed to waterpipe smoking. Group (WPS+RSV): Mice were exposed to WPS and 0.2 ml RSV, (IP) injection.

This experiment was performed and repeated daily on all animal groups for six consecutive weeks. Mice lived inside clean plastic cages. Cages were enclosed with fenestrated stainless-steel locked cover, open access to animal chew as feeding material with *ad libitum* for drinking water. Mice were acclimatized for one week prior to starting the experiment and lived-in well-ventilated room at room temperature with 12/12-hours day/night cycling periods.

Smoking exposure

Each group of mice exposed to tobacco smoking (cigarette or waterpipe) wasplaced inside transparent plexiglass box ($0.6 \times 0.5 \times 0.1$ m^3) acting as inhalation chamber to perform a smoking process using modified smoking machine described by Shraideh *et al.,* (2011). Two cigarettes were lighting for each cigarette smoking group (CS and CS+RSV), whereas a waterpipe head was uploaded with 5 grams of tobacco honeyed-maassal enclosed by perforated aluminum foil, lighting with one charcoal for both waterpipe smoking groups (WPS and WPS+RSV). After placing mice inside the inhalation chamber, a vacuum pump withdraws smoke from lightened cigarette inside the chamber to provide a smoky environment for cigarette groups (each group was exposed individually to prevent mixing). Smoking procedure was 15 minutes in duration with 15 smoking

intervals (10 seconds cigarette smoking puff + 50 seconds stop puffing with adequate ventilation), each cigarette was consumed within 7 minutes. The same procedure, generated air volume, duration and smoking intervals of cigarette groups were applied on waterpipe groups too (WPS and WPS+RSV). The same waterpipe head, uploaded tobacco honeyed maassal and charcoal were used for both waterpipe groups. RSV was delivered to animals before 30 minutes before their exposure to smoking process.

Processing of trachea for light microscopy

The mice were euthanized by physical cervical dislocation. This process was done at the end of 6th week of the exposure. Trachea was washed with PBS and fixed in 10% formal saline for 72 hours.The following techniques were performed according to Suvarna *et al.,* (2018). Trachea was dehydrated with ascending concentrations of ethanol, cleared with xylene alcohol, impregnated with melted paraffin wax, embedded in paraffin and sectioned using manual rotary microtome (Leica RM2125RT, Leica Biosystems, Germany). The sections were stained with hematoxylin (HEMHP-OT-1L, BioGnost, Croatia) for 15 minutes and eosin Y 0.2% aqueous (EOY-02-OT-1L, BioGnost, Croatia) for 5 minutesafter deparaffinization (xylene) and gradual rehydration process. After staining, the sections were dehydration using ascending concentration of ethanol alcohol prior to clearing with xylene alcohol, mounted with Dibutyl-phthalate Polystyrene Xylene (DPX) and microphotographed by (Leica inverted light microscopy, Leica Microsystems, Germany) installed with colored digital camera (Leica EC3, Switzerland) and monitored manually by computer software (Leica Application Suite LAS EZ version 1.8.0, Leica Microsystems, Switzerland).

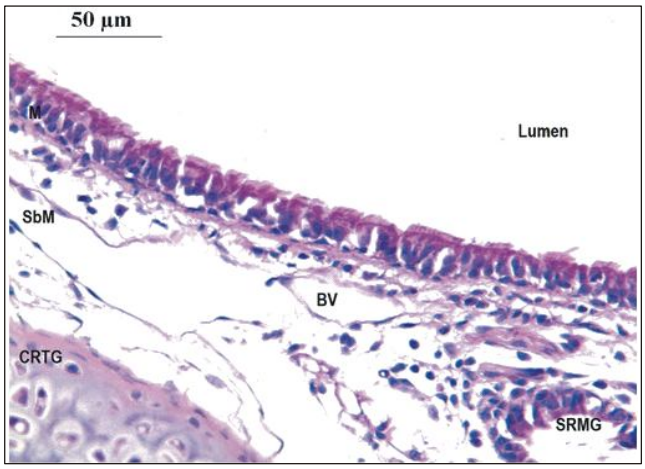

Fig. 1: Cross section of trachea for control group in mice stained with hematoxylin and eosin. Blood vessel (BV); Cartilage (CRTG); Mucosa layer (M); Submucosal glands (SRMG); Submucosal layer (SbM). Magnification X400; Scale bar 50 µm; $n = 8$.

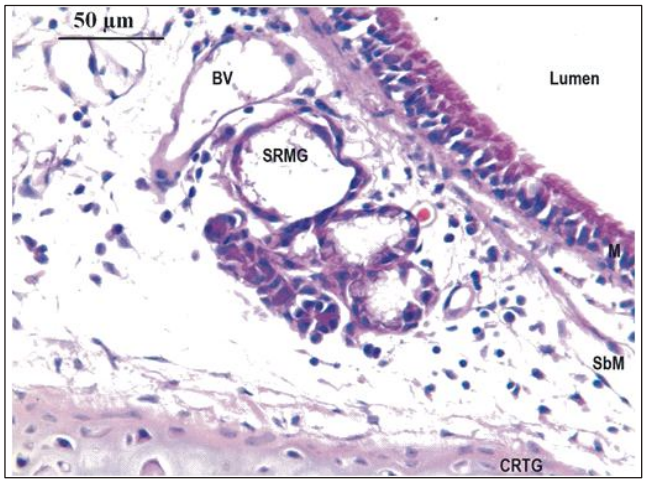

Fig. 2: Cross section of trachea for resveratrol group in mice stained with hematoxylin and eosin. Blood vessel (BV); Cartilage (CRTG); Mucosa layer (M); Submucosal glands (SRMG); Submucosal layer (SbM). Magnification X400; Scale bar 50 µm; $n = 8$.

Results

The epithelial lining of the trachea in the control group showed normal pseudostratified columnar epithelium with cilia projecting into the lumen. It was evident that submucosal glands look normal and the connective tissue was typically distributed with minimal leukocytes infiltration (Fig. 1).

RSV group showed the same histological features of the control group. It showed normal epithelial cells, normal submucosal glands and minimal leukocytes infiltration (Fig. 2).

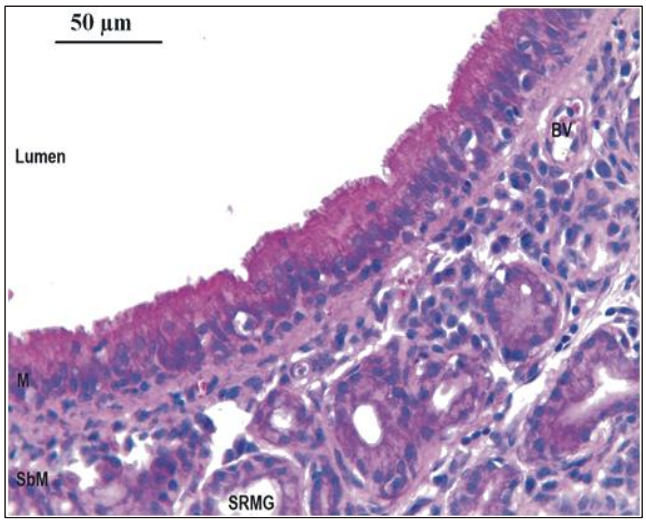

Fig. 3: Cross section of trachea for cigarette smoking group in mice stained with hematoxylin and eosin. Blood vessel (BV); Cartilage (CRTG); Mucosa layer (M); Submucosal glands (SRMG); Submucosal layer (SbM). Magnification X400; Scale bar 50 µm; $n = 8$.

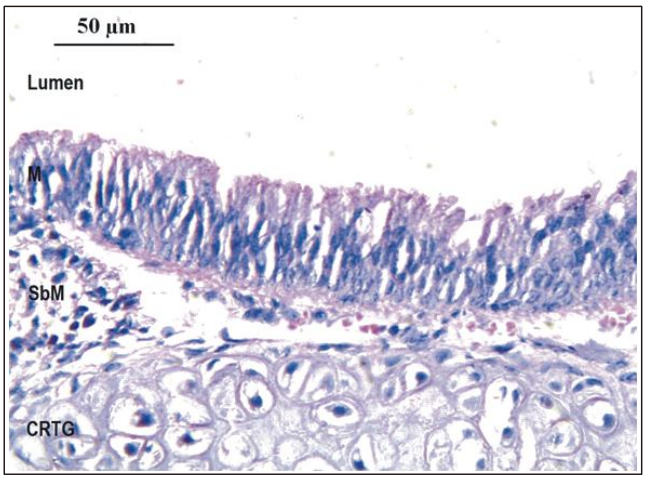

Fig. 4: Cross section of trachea for resveratrol + cigarette smoking group in mice stained with hematoxylin and eosin. Blood vessel (BV); Cartilage (CRTG); Mucosa layer (M); Submucosal glands (SRMG); Submucosal layer (SbM). Magnification X400; Scale bar 50 µm; $n = 8$.

CS group showed thick mucosa with darker epithelial cells. The epithelial cells showed disrupted cilia, some inclusion bodies, hyperplasia of basal cells and hypertrophy of submucosal glands (Fig. 3). Connective tissue fibers were partialy distributed among the submucosal layer. High infiltration of inflammatory cells but blood vessels appeared normal without dilation or congestion in submucosal layer (Fig. 3).

CS+RSV showed less disturbance in epithelial organization, less disrupted longer cilia than CS group (Fig. 4). Connective tissue fibers appear slight coherent, less hypertrophy of submucosal glands and less infiltration of inflammatory cells. No difference in hyperplasia of basal cells (Fig. 4).

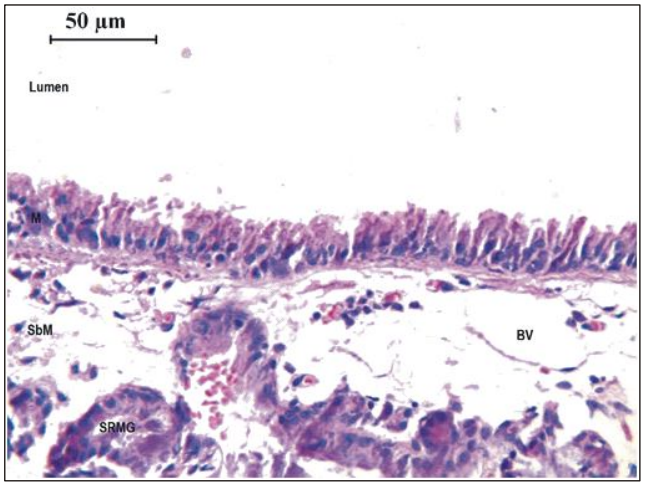

Fig. 5: Cross section of trachea for waterpipe smoking group in mice stained with hematoxylin and eosin. Blood vessel (BV); Cartilage (CRTG); Mucosa layer (M); Submucosal glands (SRMG); Submucosal layer (SbM). Magnification X400; Scale bar 50 µm; $n = 8$.

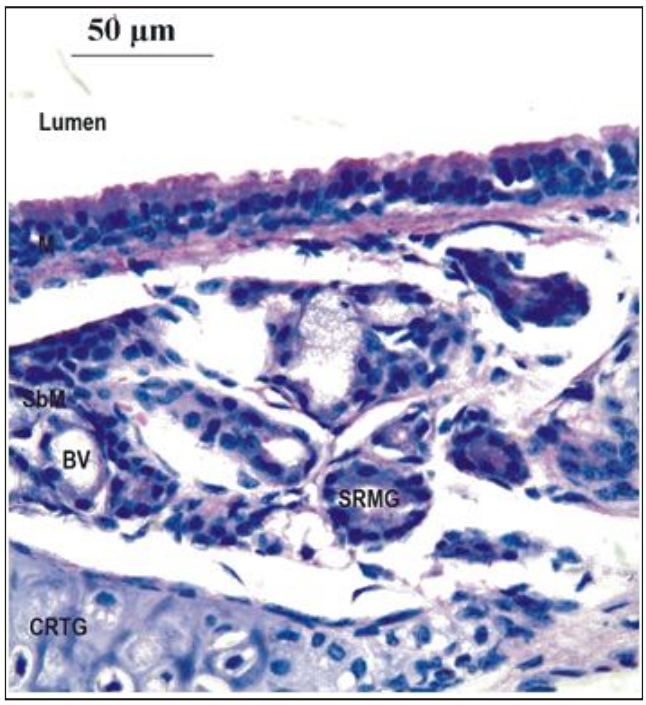

Fig. 6: Cross section of trachea for resveratrol + waterpipe smoking group in mice stained with hematoxylin and eosin. Blood vessel (BV); Cartilage (CRTG); Mucosa layer (M); Submucosal glands (SRMG); Submucosal layer (SbM). Magnification X400; Scale bar 50 µm; $n = 8$.

WPS group showed mild desquamation or cells damage in certain areas of epithelial lining, presence of some inclusion bodies and blebbing of apical membrane, lost or amalgamated cilia and hyperplasia of basal cells (Fig. 5). The submucosal layer showed hypertrophy of submucosal glands, deterioration of connective tissue, normal vascular support with slight infiltration of inflammatory cells (Fig. 5).

WPS+RSV group showed less desquamation and cells damage of epithelial lining than WPS group (Fig. 6). Also, it showed more ciliated or amalgamated ciliary projections and less inflammatory cells than WPS group. No difference in degree of hypertrophy of submucosal glands compared to WPS group (Fig. 6).

Discussion

In this study, cigarette and waterpipe tobacco smoking caused adverse effects on trachea and lung structure. Light micrographs showed disruption of the epithelial cells, hypertrophy of submucosal glands and infiltration of inflammatory cells. Previous studies showed that toxins of cigarette and waterpipe tobacco smoking have damaging effects on the mucosal and submucosal cells of the trachea. These inhaled toxins resulted in crowded epithelial cells, amalgamations of cilia, partial disruption and deteriorations in the connective tissues, hypertrophy

of submucosal glands and infiltration of inflammatory cells (Shraideh and Najjar, 2011; Imran *et al*., 2018).

Ultrastructural studies showed that tobacco smoking induces some pathological features in the trachea, illustrating that rough endoplasmic reticulum had grossly dilated cisternae containing amorphous materials at the basal region of epithelial cells. Also, tobacco smoking induces enlargement of Golgi complexes with high number of cytoplasmic vacuolization (Lewis and Jakins, 1981; Al-Awaida *et al*., 2014).

This study showed that the antioxidant RSV partially protects the epithelial cells from the toxic effects of cigarette and waterpipe tobacco smoking; displayed more extended ciliary projections with fewer amalgamations and reducing the infiltration of the inflammatory cells. It was previously proved that daily dose of RSV (10 mg/kg/day) has a prophylactic role in the trachea exposed to cigarettes smoking in albino wistar rats (Kurus *et al*., 2009).

Resveratrol has significantly decreased the level of protein oxidation in plasma by lowering the concentration of carbonyl content and increase the erythrocytic glutathione in mice induced by subchronic secondhand exposure of cigarette and waterpipe tobacco smoking (Alzbeede, 2019). Moreover, RSV showed anti-oxidant and anti-inflammatory activities against nicotine (a toxic ingredient in cigarette and waterpipe smoking) induced lung injury in rats by reducing the concentration of inflammatory biomarkers like tumor necrosis factor alpha (TNF-α), interleukin-4 (IL-4) and interleukin-6 (IL-6) (Hamza and El-Shenawy, 2017). In addition, RSV supplements has anti-inflammatory effect against multiple toxins, free radicals and oxidative stress conditions by decreasing the concentration of high sensitive C-reactive protein (hs-CRP), TNF-α and IL-6 (Koushki *et al*., 2018).

RSV has therapeutic effect on COPD mice model induced by cigarette smoking conjugated with lipopolysaccharides (a proinflammatory glycolipid in the cell wall of gram-negative bacteria). Oral administration of RSV using daily dose of 1 mg/kg or 3 mg/kg had protective and anti-inflammatory effect against acute exposure of cigarette smoking (Liu *et al*., 2014). RSV decreases the level of inflammatory cytokines like interleukin-17 (IL-17), IL-6, TNF-α and transforming growth factor beta (TGF-β) (Chen *et al*., 2016). Recent reviews and current opinions have classified RSV as a promising therapeutic candidate against COPD and different lung cancer cell lines (Yousef *et al*., 2017; Beijers *et al*., 2018).

In conclusion, this study suggests that resveratrol could has an ameliorative effect against the oxidative damage in the trachea induced by cigarette and waterpipe tobacco smoking. More researches need to be conducted in the future to investigate the role of resveratrol as an antioxidant or as an anti-inflammatory agent, especially against the toxicity of waterpipe tobacco smoking by measuring the expression in the molecular level of endogenous antioxidants and illustrating the concentrations of proinflammatory cytokine. Smoking cessation is the best solution right now to eliminate the harmful of such inhaled toxins.

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