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Chinese green tea consumption reduces oxidative stress, inflammation and tissues damage in smoke exposed rats

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ABSTRACT

Objective(s): One cause of cigarette smoking is oxidative stress that may alter the cellular antioxidant defense system, induce apoptosis in lung tissue, inflammation and damage in liver, lung, and kidney. It has been shown that Chinese green tea (CGT) (Lung Chen Tea) has higher antioxidant property than black tea. In this paper, we will explore the preventive effect of CGT on cigarette smoke-induced oxidative damage, apoptosis and tissues inflammation in albino rat model.

Materials and Methods: Albino rats were randomly divided into four groups, i.e. sham air (SA), cigarette smoke (CS), CGT 2% plus SA or plus CS. The exposure to smoking was carried out as a single daily dose (1 cigarette/rat) for a period of 90 days using an electronically controlled smoking machine. Sham control albino rats were exposed to air instead of cigarette smoke. Tissues were collected 24 hr after last CS exposure for histology and all enzyme assays. Apoptosis was evidenced by the fragmentation of DNA using TUNEL assay.

Results: Long-term administration of cigarette smoke altered the cellular antioxidant defense system, induced apoptosis in lung tissue, inflammation and damage in liver, lung, and kidney. All these pathophysiological and biochemical events were significantly improved when the cigarette smoke-exposed albino rats were given CGT infusion as a drink instead of water.

Conclusion: Exposure of albino rat model to cigarette smoke caused oxidative stress, altered the cellular antioxidant defense system, induced apoptosis in lung tissue, inflammation and tissues damage, which could be prevented by supplementation of CGT.

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Introduction

Cigarette smoking contains more than 4,000 identified chemical compounds including 60 known carcinogens (1). The gaseous components of mainstream smoke (92% of the total smoke) contain from 400 to 500 different gases. These gases include carbon- monoxide, nitrogen oxide, hydrogen cyanide, ozone and formaldehyde. Particulate matter (8% of main stream smoke) contains tar product such as naphthalene, pyrene and nitrosamine (2, 3). Also particulate matter contains metals such as polonium, cadmium, selenium, mercury, lead and arsenic (4, 5). According to Public Health Laboratories, Maryland, USA (6), the Jordanian cigarettes contain about twice the amount of nicotine and tar than non-Jordanian cigarettes.

Cigarette smoking is associated with about 400,000 deaths annually from cardiovascular diseases in the United State alone (6). There is a strong association between the degree and duration of exposure to cigarette smoking and incidence of cardiovascular events (7-11).

Cigarette smoke encompasses and creates various reactive oxygen species (ROS) and reactive nitrogen species (RNS), such as superoxide radical, hydrogen peroxide, hydroxyl radical, and peroxynitrite (12). Highly reactive radicals can damage the cell membrane and also induce DNA fragmentation, tissues damage and alter the cellular antioxidant defense system (13-14). Antioxidants, glutathione peroxidase, superoxide dismutase and catalase, can work sequentially with

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Glucose-6-phosphate dehydrogenase to dispose free radicals (14, 15).

Tea is one of the most consumed drinks worldwide where green tea (*Camellia sinensis*) accounts for about 20% of the total tea consumption (16). In recent years, several studies have shown that green tea consumption can protect against diseases that are associated with free radical damage including atherosclerosis, coronary heart disease and cancer (16-18). Because tea polyphenols have strong antioxidant properties, black tea prevents CS-induced oxidative damage of proteins both *in vitro* and *in vivo* (19). Exposing a guinea pig model to cigarette smoke caused lung damage. It appeared that oxidative stress was an initial event, followed by inflammation, apoptosis and lung injury. All those events which lead to lung damage were prevented when guinea pigs were given a black tea infusion as a drink instead of water (20). Black tea shows lower epicathechin and epigallocatechin gallate contents than green tea, which can be explained by the oxidation of its phenolic compounds (21).

Green tea contains very large amounts of catechins, including epigallocatechin gallate that accounts for more than 80% of all active ingredients in green tea and has been shown to have the greatest antioxidant activity among several compounds. The content of epigallocatechin gallate in green tea is 10-fold higher than that in black tea. Unlike black tea, green tea also contains ascorbic acid (22). Chan *et al* (23) demonstrated that increased levels of systemic oxidative stress after cigarette smoke exposure may show an important role in the induction of lung damage. This damage can be prevented by Chinese green tea consumption.

The presence of local oxidative stress and protease/anti-protease imbalance in airways after cigarette smoke exposure might be alleviated by green tea consumption through its biological antioxidant activity (24).

One cause of cigarette smoking is oxidative stress that may alter the cellular antioxidant defense system, induce apoptosis in lung tissue, inflammation and damage in liver, lung, and kidney. It has been shown that Chinese green tea (Lung Chen Tea) has higher antioxidant property than black tea. In this paper we will explore the preventive effect of Chinese green tea (Lung Chen Tea) on cigarette smoke-induced oxidative damage, apoptosis and tissues inflammation in albino rat model.

Materials and Methods

Experimntal design

Forty albino rats (*Rattus norvegicus*), with an average weight of 100 to 150 g were divided into four groups, i.e. sham air (SA), cigarette smoke (CS), Chinese green tea 2% (Lung Chen Tea) plus SA or plus CS (23). The exposure to smoking was carried out as a single daily dose of one cigarette per rat for a period of 90 days using an electronically controlled digital smoking machine (25, 33). Sham control albino rats were exposed to air instead of cigarette smoke. Tissues were collected 24 hr after last CS exposure for histology and enzyme assays (23).

The digital smoking machine

An electronically controlled smoking machine was used to expose the animals to cigarette smoke. (25). The smoking machine is composed of an electronic valve, a vacuum pump and a timer to control the sequence of puffs - and fresh air- inlet and exit in the inhalation chamber allowing enough intake of tobacco smoke and preventing oxygen deprivation in chamber. Each cycle of the smoking regimen lasts for 90 sec and consists of three successive steps, operating as follows: cigarette smoke is drawn through the inhalation chamber continuously for 30 sec. An inlet to fresh air is then opened, allowing fresh air to be introduced instead of smoke, which will be washed out of the chamber. The washing out process will also take 30 sec. In the last 30 sec, the vacuum pump will be turned off, and rats will be allowed to breathe fresh air normally.

Chinese green tea (Lung Chen) preparation

Fresh 10% CGT was daily prepared by brewing 100 g dried CGT leaves in 1000 ml hot water (not boiling) for 30 min. After cooling, either tea solution (5 ml) or tap water (5 ml) was given by oral gavage twice a day 1 hr before and after sham air (SA) or cigarette smoke (CS) exposure (23). Based upon HPLC analysis, the content of EGCG in 10 ml of 10% CGT was 36 mg, which was equivalent to the amount of drinking freely 2% Lung Chen tea daily in rats (23, 24). Using the HPLC-UV method, EGCG (epigallocatechin gallate) and EC (epicatechin) were determined in CGT extract (dry weight %) to be 0.9% and 4.2%, respectively (21).

Cell extraction

Rat tissues of liver, lung, and kidneys were excised and perfused with ice-cold perfusion solution (0.15 M KCl, 2 mM EDTA, pH-7.4). Tissues were homogenized in Tris-HCl buffer (50 mM, pH 7.4), and the homogenates were centrifuged at 10,000 g and 4˚C for 30 min to obtain supernatant. Thereafter, it was separated and then used for enzyme activities measurement and estimation of protein concentration (14).

Assay of catalase (CAT) activity

Catalase activity in the tissue supernatant was assessed following the method of Luck (26). The reaction mixture contained 0.05 M Tris-buffer, 5 mM EDTA (pH= 7.0), and 10 mM H_2O_2 (in 0.1 M potassium phosphate buffer, pH= 7.0). The supernatant was added to the above mixture in a

final reaction mixture of 3 ml. The rate of change in absorbance per minute was recorded at 240 nm. Catalase activity in terms of µmoles H_2O_2 consumed per min per mg of protein was calculated using the molar extinction coefficient of 43.6 M^{-1} cm^{-1} for H_2O_2.

Assay of glucose-6-phosphate dehydrogenase (G6PD) activity

The G6PD activity was measured by the method of Tian *et al* (27). The supernatant was added to a mixture of 1 mM $MgCl_2$, 1 mM sodium azide, 50 mM Tris-HCl buffer (pH 7.6), and 0.25 mM NADP in a final volume of 3 ml. The reaction was started in cuvette at 37˚C by adding glucose 6-phosphate (0.6 mM). The increase in absorbance per min at 340 nm was measured in a spectrophotometer. Enzyme units were expressed as number of µmoles NADPH formed using the extinction coefficient 6.22 x 10^3 M in 1-cm path at 340 nm. The levels of G6PD activity were expressed in terms of µmoles NADPH produced per min per mg of protein in crude extract.

Assay of glutathione peroxidase (GPx) activity

The GPx activity was measured using the method of Paglia and Valentine (28). One unit glutathione reductase and 1.0 mM reduced glutathione were added to 50 mM potassium phosphate buffer (pH 7.0) containing 1.0 mM sodium azide, 1 mM EDTA, and 0.2 mM NADPH. The mixture (2.9 ml) was allowed to equilibrate for 5 min at 25˚C before initiating the reaction with 0.1 ml of 2.5 mM H_2O_2. The linear activity was recorded as absorbance at 340 nm. Units of enzyme activity were expressed as µmoles of NADPH oxidized to NADP by using the extinction coefficient of 6.22 x 10^3 M^{-1} cm^{-1} at 340 nm. The levels of GPx were expressed in terms of µmoles NADPH consumed per min per mg of protein in crude extract.

Histopathological examination

Following an overnight recovery from the last smoke exposure, rats were sacrificed by ether anesthesia and tissues of interest (trachea, alveoli of the lung, aorta, and ventricles of the heart) 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 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 (14).

Sections of about 5 µm thick were obtained by a microtome (Spencer 50) and mounted on glass slides stained with hematoxylin and eosin. The prepared sections were examined and photographed using Leica microscope equipped with digital camera.

Tracking apoptotic changes in tissues

The presence of apoptotic cell death in tissue sections of trachea, alveoli of the lung, aorta, and ventricles of the heart, was examined using Dead End Colorimetric

TUNEL (TdT-mediated dUTP Nick-End Labeling) kit (Promega, USA).

TUNEL assay procedure (TUNEL kit, Promega)

The DeadEnd™ Colorimetric TUNEL System is a modified TUNEL Assay designed to provide accurate detection of apoptotic cells and can be used to assay apoptotic cell death in tissue sections. This kit system measures the nuclear DNA fragmentation, an important indicator of apoptosis. The DeadEnd™ Colorimetric TUNEL System end-labels the fragmented DNA of apoptotic cells using a modified TUNEL (TdT-mediated dUTP Nick-End Labeling) assays. Biotinylated nucleotide is incorporated at the 3´-OH DNA ends using the enzyme Terminal Deoxynucleotidyl Transferase (TdT). Horseradish-peroxidase-labeled streptavidin (Streptavidin HRP) is then bound to these biotinylated nucleotides. Bound streptavidin was then detected by hydrogen peroxide, and the chromogen, diaminobenzidine (DAB).

Protein content determination

The protein concentration in the tissue homogenates was determined using Lowry method (29).

Statistical analysis

All statistical analyses were performed using the SAS software, version 9 (SAS Institute 2002). Analysis of variance was performed using Mixed procedure (Proc Mixed) to perform a mixed model. Mean were then compared using LSD. A *P*-value < 0.05 was considered significant.

Results

Catalase activity

Cigarette smoke exposure caused a significant decrease in catalase specific activity compared to sham air exposed rats given water as a drink by 23%, 18% and 50% in liver, kidney and lung tissues, respectively. However, after CGT administration in combination with cigarette smoke exposure, the catalase specific activities were significantly increased in liver, kidney and lung tissues compared to smoke exposed rats given water as a drink. Consumption of CGT alone had no effect on catalase activity (Table 1).

Glutathione peroxidase activity

Cigarette smoke exposure caused a significant decrease in glutathione peroxidase specific activity compared to sham air exposed rats given water as a drink (16%, 13% and 35% in liver, kidney and lung

Table 1. Catalase, glutathione peroxidase and G6PD activity in liver, kidney and lung tissues of different groups of rats

Tissues	Sham air exposed rats given water as a drink ¥	Smoke exposed rats given water as a drink ¥	Sham air exposed rats given green tea as a drink ¥	Smoke exposed rats given green tea as a drink¥	Critical LSD value
Catalase activity [1]					
Liver	31.67a*	24.33c	30.85a	27.87b	1.77
Kidney	8.95 a	7.3c	9.15a	8.5b	0.50
Lung	1.95 a	0.96c	1.85a	1.43b	0.16
Glutathione peroxidase activity [2]					
Liver	86e	72.67g	87.15e	78.26f	4.1
Kidney	53e	45.3f	51.9e	50.88e	2.7
Lung	35e	22g	36e	28f	2.4
G6PD activity [3]					
Liver	3.4h	2.4j	3.5h	2.96i	0.19
Kidney	3.7h	3.5i	3.55h	3.44h	0.25
Lung	2.27h	1.35j	2.15h	1.89i	0.17

* For each tissue type, the same letter means the values are not significantly different according to LSD (0.05 probability level)
[1] The level of catalase activity was expressed in terms of µmoles H_2O_2 consumed/min /mg of protein in crude extract, [2] The levels of glutathione peroxidase activity were expressed in terms of µmoles NADPH consumed/min/ mg of protein in crude extract, [3] The levels of G6PD activity were expressed in terms of µmoles NADPH produced/min /mg of protein

tissues, respectively). However, after CGT administration in combination with cigarette smoke exposure, the glutathione peroxidase specific activities were significantly increased in liver, kidney and lung tissues compared to smoke exposed rats given water as a drink. Consumption of CGT alone had no effect on glutathione peroxidase (Table 1).

Glucose-6-phosphate dehydrogenase (G6PD) specific activity

The cigarette smoke exposure caused a significant decrease in G6PD specific activity compared to sham air exposed rats given water as a drink by 29 %, 18% and 38% in liver, kidney and lung tissues, respectively. However, after CGT administration in combination with cigarette smoke exposure, the G6PD specific activities were significantly increased in liver, kidney and lung tissues compared to smoke exposed rats given water as a drink. Consumption of CGT alone had no effect on glucose-6-phosphate dehydrogenase (Table 1).

Histological examination

Compared to normal morphology of liver, lung, and kidney of air-exposed rat given water as a drink as shown in (Figure 1A). Long period smoking exposure of rats, produced inflammatory changes in liver, lung, and kidney tissues. These changes involved interstitial inflammation involving lymphocytes and plasma cells in lung (Figure 1B1), portal area inflammation in liver (Figure 1B2) and mesangial cell proliferation in corpuscles (Figure 1B3). All these inflammatory changes were significantly improved when the cigarette smoke-exposed albino rats were given CGT infusion as a drink instead of water (Figure 1C). CGT itself did not have such effect (Figure 1D).

Chinese green tea (Lung Chen Tea) prevents cigarette smoke induced apoptosis in rat lung sections

Compared to lung tissue from air-exposed rats given water as a drink as shown in (Figure 2A). Green tea itself did not induce apoptosis in the rat lung (Figure 2B). Cigarette smoke induced apoptosis only in lung section. There were dark-brown apoptotic nuclei in the examined sections (Figure 2C). Our result indicated that supplementation of CGT prevents cigarette smoke induced apoptosis in the rat lung (Figure 2D).

Discussion

Long-term administration of cigarette smoke induces apoptosis in lung tissue, tissues injury and impairs the enzymatic antioxidant defense system in liver, lung, and kidney. All these pathophysiological and biochemical events were significantly improved when the cigarette smoke-exposed albino rats were given green tea infusion as a drink instead of water. The obtained data also suggests different effects of cigarette smoke on different organs which could be due to the differential load of metabolites of cigarette smoke in these organs (23, 30). That can change the antioxidant defense system leading to an oxidative stress with a variable effect on these organs.

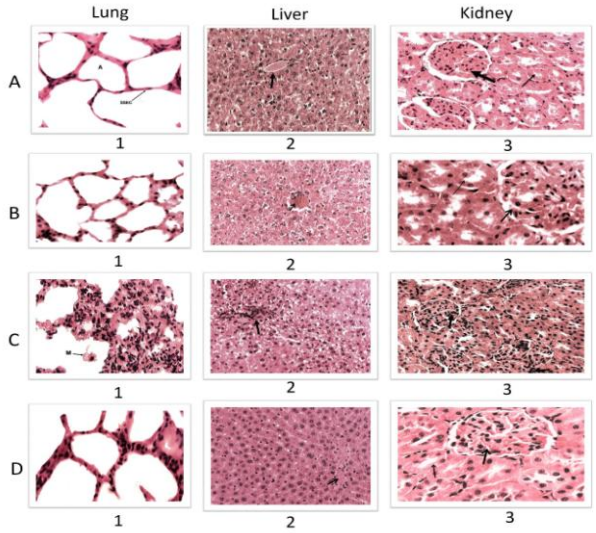

Figure 1. Consumption of Chinese green tea effect (Lung Chen) on tissue histologyby using hematoxylin and eosin stain (H & E). A. Normal morphology of liver, lung, and kidney of air-exposed rat given water as a drink (sham control). B. Morphology of liver, lung, and kidney of air-exposed rat given CGT as a drink. C. Morphology of liver, lung, and kidney of cigarette smoke-exposed rat given water as a drink (1) lung tissue shows some degree of collapsed alveoli and inflammatory cell infiltration. M: Macrophage, (2) liver tissue shows portal area inflammation in liver. Arrow shows phagocytic cells, and (3) kidney tissue shows mesangial cell proliferation. Arrow shows mesangial cell. D. Morphology of liver, lung, and kidney of cigarette smoke-exposed rat given CGT as a drink (1) lung tissue shows less thickening in the alveolar wall and no inflammatory cell infiltration was observed, (2) liver tissue shows the absence of portal area inflammation. Arrow shows portal area and (3) kidney tissue shows absence of mesangial cell proliferation in the renal corpuscle. Thick arrow shows Renal corpuscle and thin arrow shows proximal tubule

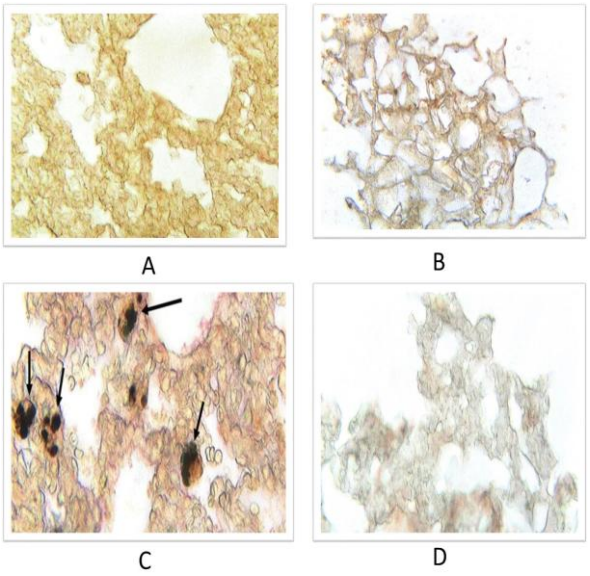

Figure 2. Detection of apoptotic nuclei in rats' lung cells exposed to air or CS in either presence or absence of CGT by TUNEL assay. A. rats exposed to air and given water as a drink. B. rats exposed to air and given CGT as a drink. C. rats exposed to CS and given water as a drink. The arrow indicates one apoptotic nucleus. D. rats exposed to CS and given CGT as a drink instead of water

An imbalance between cellular pro-oxidant and antioxidant levels leads to the oxidative stress resulting in tissue damage. The antioxidant enzyme interacts directly with reactive oxygen species (ROS) to convert them to non-radical products. We had previously demonstrated that the overproduction of these radicals by cigarette smoking has an inhibitory effect on the enzymes responsible for removal of ROS such as CAT, G6PD and GPx (33). The toxicity of aqueous extract of smokeless tobacco (AEST) in different organs like liver, lung, and kidney is due to the formation of the free radical species (14), which might explain the inhibition of these enzymes after the long-term administration of cigarette smoke in the present study.

In correlation with the alterations in enzymes activities, present data demonstrated an induction of inflammatory changes in tissues due to the effect of chronic smoking exposure on rats. These abnormality changes involved an interstitial inflammation (the presence of lymphocytes and plasma cells in lungs), portal tract inflammation in liver and the proliferation of mesangial cells in kidney corpuscles. All these inflammatory changes were significantly improved when the cigarette smoke-exposed albino rats were given green tea infusion as a drink instead of water. It has been reported that prolonged smoking causes deficiencies in antioxidant enzyme activities of different tissues in rat (14). This impairment was due to the inhibitory effects of ROS present in tobacco constituents on these enzymes. Also, tobacco smoke has many toxic low molecular weight compounds that are able to directly stimulate bronchoalveolar dendritic cells (DCs). Activated DCs can discharge many of inflammatory chemokines that will induce the recruitment and activation of more DCs and other inflammatory cells such as neutrophils (34).

Tobacco smoking also induced dose-dependent increase in goblet cell formation and mucin secretory ability of rat airways (35). Activation of neutrophils, in turn, released a number of mediators and proteases that spread the inflammatory response and contribute to the destruction of the lung airways. It was shown that the long-term administration of tobacco smoking can impair the enzymatic antioxidant defense system of the rat liver, lung, and kidney. These alterations may be one of the responsible factors for smoking-induced inflammation in these organs (33).

Long-term administration of cigarette smoke caused apoptosis in lung tissue and impaired the enzymatic antioxidants in liver, lung, and kidney. Such alterations may lead to the cigarette smoke induced inflammation in these organs. Kuo *et al* (36) demonstrated that the effect of CS-induced lung injury including apoptosis may be via reactive oxygen species and nitrogen oxides generation. The formation of these oxidizing agents leads either to the phosphorylation of

p38/JNK MAPK pathway and then activation of Fas cascades, or to stimulate the stabilization of p53 and increase in the ratio of Bax/Bcl-2. Polyphenols in black tea have antioxidant properties preventing CS-induced oxidative damage in both *vitro* and *vivo* (19). When the exposed guinea pig model to cigarette smoke was given black tea, the oxidative stress was reduced (20). Because of the oxidation of phenolic compounds in black tea, the green tea shows higher epicathechin and epigallocatechin gallate content (21).

Increased levels of systemic oxidative stress after cigarette smoke exposure may show an important role in the induction of lung damage. CGT has a protective effect on cigarette smoke induced suppressive effects on lung tissue and local oxidative stresses (23).

The protective role of green tea against cigarette smoke induced oxidative stress may be explained by the large amounts of catechins, including epigallocatechin gallate that accounts for more than 80% of all active ingredients in green tea and has been shown to have the greatest antioxidant activity among several compounds. The content of epigallocatechin gallate in green tea is 10-fold higher than that in black tea. Unlike black tea, green tea also contains ascorbic acid. These results suggest that green tea may act as a potent antioxidant (22). So, the results obtained with albino rats in our present study would imply that regular intake of Chinese green tea (Lung Chen) may protect smokers from the risk of developing tissues damage.

Conclusion

Exposure of albino rat model to cigarette smoke caused oxidative stress, altered the cellular antioxidant defense system, induced apoptosis in lung tissue, inflammation and tissues damage, which could be prevented by supplementation of Chinese green tea (Lung Chen Tea).

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