

## Ameliorative Effect of Turmeric and Cocoa Extract against Acute Second Hand Exposure of Tobacco Smoking on Hepatocytes and Enterocytes in Albino Rats: Ultrastructural Study

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Smoking posse's serious health problems because there is no specific substance alleviate its toxicity. The aim of this study was to investigate the effective plant extract containing antioxidants(turmeric and cocoa), that may reduce the cytotoxicity induced by secondhand exposure of cigarettes and water pipe smoking. Seventy-two adult male albino rats were equally divided into 9 group (n=8 per group). Extracts were delivered to each group intraperitoneal, and the exposure to cigarette and water pipe smoking was performed using a smoking machine for a period of 30 days. After the exposure period, tissues of interest (liver and small intestine) were removed and processed for transmission electron microscopy. Oral treatment of turmeric and cocoa extract with smoking exposure showed less vacuolization and better cellular architecture with regular nuclear envelope of hepatocytes, reduce or absence of blebbing, retain the normal shape and size of mitochondria, increase the proportion of euchromatic chromatin in nuclei, but turmeric extract showed better enhancement in term of reducing vacuolization. turmeric or cocoa crude extract preserve the typical length of microvilli with a uniform organization from the apical part of enterocytes and decreased vacuolization in the cytosol of enterocytes. However, mitochondria appeared less polymorphic in shapes with distinct cristae and matrix in enterocytes of the turmeric treated group than the cocoa once. Tobacco smoking-induced adverse effects on hepatocytes and enterocytes, this study showed that treatment with turmeric and cocoa attenuatethe toxicity of tobacco smoking.

**Keywords:** Antioxidant; Microscopy; Histopathology; TEM; Tobacco smoking.

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Tobacco leaves are smoked as cigarettes, chewed or used in waterpipes (also known as narghile, or arghileh shisha, goza, hookah, and

hubbly bubbly)<sup>1</sup>. It has been estimated that tobacco smoking was responsible for more than seven million deaths each year<sup>2</sup>, and it is expected that by

2030 the death rate will exceed millions per year in developing countries<sup>3</sup>.

Many toxicants found in waterpipe smoking such as tar, carbon monoxide, heavy metal<sup>1,4</sup>. The presence of these toxic substances in different concentrations may cause major lethal illnesses, including lung cancer, cardiovascular disease and chronic obstructive pulmonary disease<sup>5</sup>. Secondhand exposure to cigarette and waterpipe tobacco smoking can induce histopathological changes and inflammation on different organs in the experimental animals<sup>6-8</sup>. Nicotine, a constituent of tobacco, is reported to induce oxidative stress both in vitro and in vivo, as well as depleting antioxidant defense mechanisms<sup>9</sup>.

There are several important natural antioxidants which have a protective effect against free radicals<sup>10, 11</sup>. Natural antioxidants with free radical-scavenging activity such as the cocoa bean have received much attention, due to their health-promoting properties. Cocoa is the mature fruit of the cacao tree (*Theobroma cacao L.*), which grows in tropical regions of Africa and South America<sup>12</sup>. The main components of cocoa powder that have an antioxidant capacity are polyphenol and methylxanthine compounds, where these could significantly contribute to their health-promoting activities<sup>13</sup>. Cocoa beans are one of the main sources of polyphenols, especially epicatechin. Epicatechin has been reported to have an antioxidant capacity<sup>14</sup>. High consumption of chocolate and cocoa was associated with a decrease in both blood pressure and the risk of cardiovascular disease<sup>15</sup>.

Turmeric (T) is a yellow powder, derived from the plant *Curcuma longa*. Pharmacological studies have demonstrated that it has anti-tumor, anti-inflammatory, anti-infection, and antioxidant activity, with very low toxicity<sup>16</sup>. Turmeric consists of a water-soluble component, turmeric (T; mol weight 24,000 daltons), and lipid-soluble component Curcumin (Cu; mol weight 369.89 daltons). Curcumin polyphenols (diferuloylmethane) inhibit toxin-mediated stress responses via their anti-inflammatory and antioxidant properties in addition to inducing the expression of cytoprotective proteins<sup>17</sup>. Curcumin neutralizes nitric oxide, provides continuity to antioxidant enzymes such as catalase and superoxide dismutase, and lowers lipid

peroxidation, to protect tissues and organs from oxidative damage caused by nicotine<sup>18</sup>.

This study aims to assess the ultrastructural alterations of the liver and small intestine of albino rats induced by two type of tobacco smoking; and investigate the effect of using turmeric and cocoa extract to protect these two organs from damage induced by cigarette or waterpipe tobacco smoking.

## MATERIALS AND METHODS

### Preparation of turmeric and cocoa extract

Cocoa blend and curcumin from *Curcuma longa* (turmeric) powder were purchased from (Sigma, Germany). Both antioxidants containing materials were macerated in 80% methanol for three days, according to<sup>19</sup>, then filtered and concentrated to a small volume to remove the entire methanol using a rotary evaporator. The low volume was later freeze-dried to obtain a crude extract and kept at 4°C until needed. The cocoa or curcumin crude extract were administered to rats via oral gavage with a dose of 200 mg/kg/day<sup>19</sup>.

### Experimental design in vivo

Seventy-two adult male albino rats were purchased from animal's house / Jordan University of Science and Technology, Irbid, Jordan. Rats were randomly divided into nine groups; each contains eight rats, and left to acclimatized in room temperature, well-ventilated room with light/dark cycle of 12/12 hours, feed with standard animal chew and free-water access *ad libitum*.

This experiment was performed in Animals House / Department of Biological Sciences / The University of Jordan. All animal experiments were conducted according to the guidelines of European Commission Directive 86/609/EEC for laboratory animal care and use. The experimental groups were divided as following:

Group 1 (Control): Rats were exposed to fresh air only.

Group 2 (Curcumin): Rats were uptake curcumin via oral gavage with dose of 200 mg/kg/day.

Group 3 (Cocoa): Rats were uptake cocoa crude via oral gavage with dose of 200 mg/kg/day.

Group 4 (Cigarette smoking): Rats were exposed to cigarette smoking (1 cigarette/rat/day).

Group 5 (Cigarette smoking + Curcumin): Rats were uptake curcumin with dose of 200 mg/kg/day before one hour from exposure to cigarette

smoking (1 cigarette/rat/day).

Group 6 (Cigarette smoking + Cocoa): Rats were uptake cocoa crude extract with dose of 200 mg/kg/day before one hour from exposure to cigarette smoking (1 cigarette/rat/day).

Group 7 (Waterpipe smoking): Rats were exposed to waterpipe smoking (1 waterpipe tobacco head 5 g molasses/rat group/day).

Group 8 (Waterpipe smoking + Curcumin): Rats were uptake curcumin with dose of 200 mg/kg/day before one hour from exposure to waterpipe smoking (1 waterpipe tobacco head 5 g molasses/rat group/day).

Group 9 (Waterpipe smoking + Cocoa): Rats were uptake cocoa crude extract with dose of 200 mg/kg/day before one hour from exposure to waterpipe smoking (1 waterpipe tobacco head 5 g molasses/rat group/day).

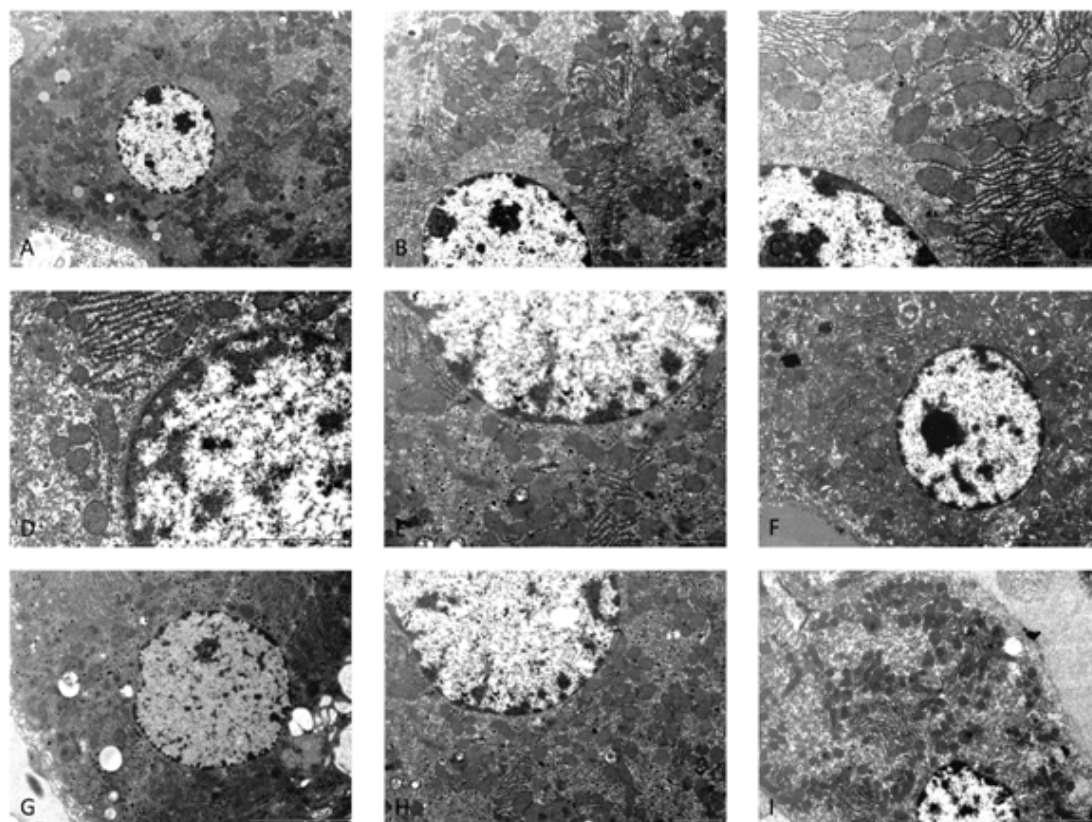
The cigarettes (L&MRed, Philip Morris, Jordan) and tobacco molasses (two apples flavor,

Mazaya, Jordan) were purchased from local markets.

Each group of rats exposed to tobacco smoking (cigarette or waterpipe) was placed inside transparent plexiglass box (0.6 x 0.5 x 0.1 m<sup>3</sup>) acting as inhalation chamber to perform a smoking process using modified smoking machine described by [4]. The duration for experimental exposure lasts for 30 consecutive days.

All rats were euthanized by cervical dislocation and dissected to obtain the liver and parts of small intestine. Those organs were washed properly using phosphate buffered saline pH 7.2, 2.5 % glutaraldehyde for 72 hours prior to electron microscopy preparations.

Tissues of the liver, small intestine were cut into approximately (1 mm<sup>3</sup>), then fixed with 2.5 % glutaraldehyde in PBS (0.1 M, pH 7.2) at 4 °C in glass vials for 72 hours. The tissue post-fixed with 1% osmium tetroxide in in PBS for 1 hr according



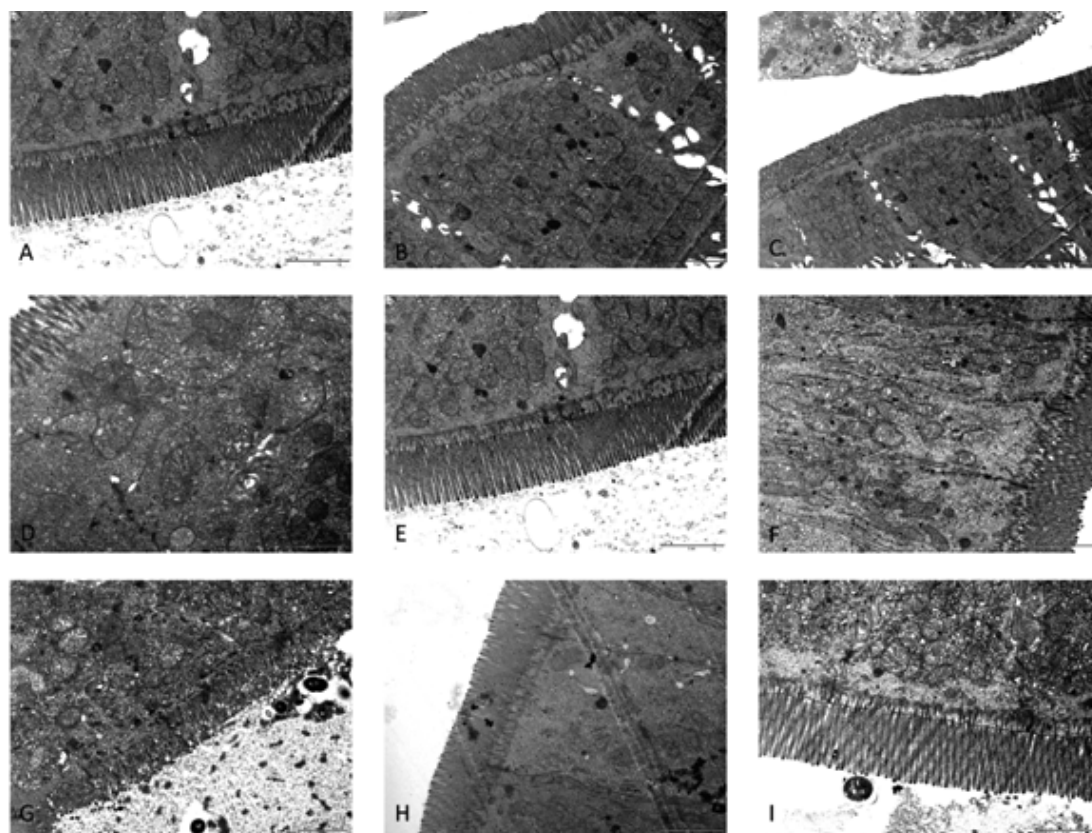
**Fig. 1.** Ultrathin sections of 70 nm from liver of different experimental groups. (A-C) control group, (D) cigarette Smoking, (E) cigarette smoking + turmeric, (F) cigarette smoking + cocoa, (G) waterpipe smoking, (H) waterpipe smoking + turmeric, (I) waterpipe smoking + cocoa. Sections were stained with uranyl acetate and counter-stained with lead citrate; electron micrographs under low magnification

to [20]. Dehydration was done by immersing the samples for 5 min ascending grades of ethanol. The tissues were cleared by pure propylene oxide (110205, Sigma Aldrich, USA) with continuous rotation for 30 minutes, and then infiltrated with 1:1 propylene oxide:epoxy resin mixture with continuous rotation at room temperature for at least 3 hours. Eventually, the tissues blocks were embedded in conical beam embedding capsules with pure epoxy resin and polymerized for 24 hours in oven at 60 °C. Golden ultrathin sections (70 nm) were prepared using diamond knife (DiATOME Ultra Diamantesser 45°, Switzerland) installed on ultramicrotome (Leica EM UC7, Leica MicroSystems, Germany). The ultrathin sections were mounted on copper grids (200 mesh grids, each about 97 µm) and stained with uranyl acetate and counter-stained with lead citrate. The grids were

examined using transmission electron microscope (*Morgagni 268*, FEI Philips, Netherlands), monitored through computer software (*Morgangi 268D* - Version 2.21, Netherlands). The ultrathin sections were photographed at 60 KiloVolts at different magnifications.

## RESULTS

Ultrathin sections from the control, turmeric, and cocoa extract of experimental rats showed normal hepatocytes containing normal large, round nuclei with predominant euchromatin. Mitochondria, which filled the cytoplasm, were normal in shape and size containing visualized cristae. Abundant rough endoplasmic reticulum was normally distributed within the cytosol of hepatocytes (Fig. 1A-C).



**Fig. 2.** Ultrathin sections of 70 nm from small intestine of different experimental groups. (A-C) control groups, (D) cigarette Smoking, (E) cigarette smoking + turmeric, (F) cigarette smoking + cocoa, (G) waterpipe smoking, (H) waterpipe smoking + turmeric, (I) waterpipe smoking + cocoa. Sections were stained with uranyl acetate and counter-stained with lead citrate; electron micrographs under low magnification

However, rats treated with cigarette smoking showed hepatocytes with polymorphic mitochondria with mild deterioration in cristae and matrix; nuclei contain some chromatin condensations adjacent to the inner margins of the nuclear envelope, the nuclear envelope appeared irregular in shape, rough endoplasmic reticulum appeared scattered and dilated cisternae, the cytosol content was distorted with the presence of some vacuolization (Fig. 1D). Oral uptake of turmeric extract with cigarette exposure showed less vacuolization and better cellular architecture with regular nuclear envelope of hepatocytes in comparison with cigarette smoking only (Fig. 1E). Parallel treatment of Cocoa crude extract along with cigarette smoking, yet vacuolization presents with disrupted mitochondria (Fig. 1F).

The examined ultrathin section of hepatocytes in mice exposed to waterpipe smoking showed several abnormalities; increase in the number of mitochondria, and swollen in shape, thinning with disorganization of mitochondrial cristae, and the nucleus has heterochromatic DNA with intact nucleus envelope. Vacuolization and lipid droplets were evident, and some blebs were displayed from the plasma membrane of hepatocytes (Fig. 1G). Ultrathin sections of liver tissue from waterpipe smoke-exposed rats with turmeric showed better recovery results in hepatocytes in comparison to sections of hepatocytes of cocoa extract treatment with waterpipe tobacco smoking (Fig. 1H & I). The treatment with these crude extracts showed some similarities like reduce or absence of blebbing, retain the normal shape and size of mitochondria, increase the proportion of euchromatic chromatin in nuclei. But turmeric extract showed better enhancement in term of reducing vacuolization.

Ultrathin sections from the control, turmeric, and cocoa extract of experimental rats (Fig. 2 A-C) showed normal morphology of enterocytes containing typical withstanding microvilli, rod-shaped mitochondria, and no abnormalities were noticed in their organelles.

The cigarette smoking exposed group showed atypical cellular morphology; mitochondria appear in polymorphic shapes containing partial disorientation in their cristae, and the matrix appeared to be diffused and electron-lucent (Fig 2D). Also, it showed a slight shortening

of microvilli, the organelles contained granular electron-dense inclusions, and vacuolization was observed in the cytoplasm of enterocytes. Parallel treatment with turmeric or cocoa crude extract (Fig. 2 E-F) preserve the typical length of microvilli with a uniform organization from the apical part of enterocytes and decreased vacuolization in the cytosol of enterocytes. However, mitochondria appeared less polymorphic in shapes with distinct cristae and matrix in enterocytes of the turmeric treated group than the cocoa once.

In waterpipe tobacco smoking, ultrathin sections of the enterocytes were seen to have edema and with slight deformity and dilation of cellular membranes resembling blebs. They contain excessive dense bodies with small vacuoles of lipidic nature distributed within the cytoplasm; rounded mitochondria with deteriorated cristae; microvilli baselines were irregular and shortening in their lengths. The enterocytes of this group slightly lost their sinuosity due to cellular swelling with the indication of moderate to severe structure injury (Fig. 2G). Turmeric or cocoa crude extract treatment show (Fig. 2 H-I) uniform of microvilli length organization and decreased in vacuolization in cytosol of enterocyte with less polymorphism of mitochondria, these appear in turmeric treatment more than cocoa.

## DISCUSSION

Tobacco causes many of the world's major lethal illnesses, including chronic obstructive pulmonary disease, lung cancer, and cardiovascular disease<sup>21</sup>. The rising prevalence of smoking worldwide with its related risks made it mandatory to find remedies to ameliorate its serious consequences<sup>22</sup>. In this research, we study the effect of use natural products containing antioxidants (cocoa and turmeric) against the ultrastructural alterations in liver and small intestine in experimental rats induced by cigarette and waterpipe tobacco smoking.

This study showed that turmeric had chemopreventive ability to attenuate the oxidative damage induced by cigarette and waterpipe smoking through retaining the normal architecture of hepatocytes, decreasing the vacuolization within these cells, our results demonstrated pleomorphic mitochondria with electron dense condensations

inside them and partially disrupted cristae. In addition, microvilli appeared disrupted. The changes in the mitochondria might explain the oxidative stress in the jejuna enterocytes.

Exposure to cigarette smoke, may affect both the function and structure of intestinal barrier, as it increases intestinal permeability, causes damage of microvilli, leads to bacterial translocation and abnormal tight junction proteins with destruction of tight junction<sup>23</sup>. Microvilli aid in transport molecule to blood stream and increase the surface area, contribute to absorption process, the destruction or any abnormality in microvilli structure or function may cause inflammation that effect absorption and breakdown of nutrient, which will result in malabsorption of nutrients.

Turmeric neutralizes nitric oxide, provides continuity to antioxidant enzymes such as catalase and superoxide dismutase, and lowers lipid peroxidation, to protect tissues and organs from oxidative damage caused by nicotine<sup>18</sup>. Previous studies stated that turmeric has gastroprotective effect the ability of turmeric to rises mucin secretion also minimize the gastric ulcer complications<sup>24</sup>. Our results showed that the impact of enterocyte with less vacuolization in cytosol, and the microvilli uniform in shape with more organization in apical part of enterocyte, the mitochondria less polymorphism with normal cristae.

Cocoa beans are one of the main sources of polyphenols, especially epicatechin. Epicatechin has been reported to have an antioxidant capacity<sup>14</sup>. These polyphenols content in cocoa have anti-mutagenic, anti-inflammatory and anti-cariogenic effects<sup>25,26</sup>.

Our result show that the treatment by cocoa extract of small intestine exposure to smoking the microvilli appear parallel, long on the apical part of epithelia cell, mitochondria with various shape cristae are prominent and decreased of vacuolization in cytosol of enterocyte.

Our results demonstrated using tobacco causes adverse impact in hepatocyte, mitochondria which appeared swollen and polymorphic shape with mild deterioration in cristae and matrix; and nucleus heterochromatic with irregular shape of nucleus envelope.

Rough endoplasmic reticulum shows dispersed and dilated cisterns, the cytosol content

was blurred by the presence of some vacuolation. These results were consistent with earlier findings that presented exposure of rat for three months to cigarette smoking lead to decrease of matrix density with disrupted of mitochondrial cristae, increase size of endoplasmic reticulum, and rise and regular nuclear envelope in comparison with cigarette smoking only. while treatment with cocoa extract showed less recovery effect in hepatocyte than turmeric extract, still destruction and in number of mitochondria<sup>20</sup>. Treatment with turmeric extract with cigarette exposure showed less vacuolization and improved in hepatocyte structural with normal mitochondria pleomorphic shape of mitochondria.

Many study showed the protective effect of turmeric again toxic component such as copper, cadmium, mercury and lead which elevate heavy metal, the turmeric minimize the hepatotoxicity and maintain against mitochondrial dysfunction<sup>27</sup>. Also turmeric can have as antioxidant anti-inflammatory and antimicrobial effect<sup>28</sup>. Turmeric neutralizes nitric oxide, provides continuity to antioxidant enzymes such as catalase and superoxide dismutase, and lowers lipid peroxidation, to protect tissues and organs from oxidative damage caused by nicotine<sup>18</sup>.

## CONCLUSION

Our results showed that rats exposed to water pipe smoking, indicate extreme histological changes in mitochondria structure the appear swollen in shape and increase in number with disorganization of cristae, and the nucleus has heterochromatic DNA with intact nucleus envelope. This study demonstrated that the using of turmeric extract with waterpipe smoke to keep the hepatocyte architecture and normal structure of mitochondria also cell membrane in its correct state, While the use of cocoa extract reduced the cell-destructive effect of smoking toxicity. The main components of cocoa powder that have an antioxidant capacity are polyphenol and methylxanthine compounds, where these could significantly contribute to their health-promoting activities.

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#### Disclosure

The authors declare that there is no conflict of interests regarding the publication of this article.

#### Copyright Transfer

This manuscript has not been published or submitted for publication elsewhere.

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