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**Research article**

# Protective effect of thymoquinone on smoking-induced vascular damage: An experimental study in rats

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## ABSTRACT

**Aim:** To investigate the protective effect of thymoquinone on smoking-induced vascular damage in rats.

**Method:** A total of 28 rats were allocated in this experimental study. Rats were equally divided in four groups; as control group (n=7) and study groups including only smoking group (n=7), smoking plus thymoquinone group (n=7) and smoking plus dexamethasone group (n=7). The animals in study groups were subjected to regular cigarette smoke exposure during 2 months, hereby smoking-induced vascular injury model was established in the animals. The thoracic aorta of the animals were surgically resected and then evaluated histopathologically. The prepared aortic tissue samples were analyzed under light microscope in terms of thickness of aortic wall, endothelial continuity and injury as well as degenerative alterations on the endothelium.

**Results:** Mean aortic wall thicknesses were 77.8 and 114.5 μ in only smoking group and smoking plus thymoquinone group respectively, and this difference was statistically significant. In only smoking group partial endothelial damage and complete endothelial damage were observed in 2 and 4 animals respectively whereas in smoking plus thymoquinone group no endothelial damage and partial endothelial damage were observed in 2 and 4 animals respectively. When these groups were compared in terms of endothelial damage, the difference was detected to be statistically significant.

**Conclusion:** Our study demonstrates that thymoquinone has a protective effect on rat endothelium and alleviates the smoking-induced vascular damage in rats.

**Keywords:** Thymoquinone, smoking-induced vascular damage, experimental, rat.



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## Introduction

Cigarette smoking is one of the most important independent risk factors in the development of cardiovascular diseases [1]. World Health Organization predicts yearly deaths due to cigarette smoking will raise to 8 million by 2030 [2]. It is known that cigarette smoking directly contributes the atherogenesis especially on endothelial cells. Cigarette smoking-induced endothelial dysfunction is triggered by the decreased nitric oxide (NO) bioavailability and increased expression of adhesion molecules. Cigarette smoking-induced increased adherence of platelets and macrophages leads to the occurrence of an inflammatory and procoagulant environment [1]. In various experimental studies, cigarette models in animals has been established, and many molecules have been researched to alleviate cigarette smoking-induced vascular injury in experimental animals. Due to its antiinflammatory, antioxidant and immunomodulatory properties, thymoquinone (2-isopropyl-5-methylbenzo-1,4-quinone) has become a intriguing and popular substance for the traditional treatment of a wide spectrum of diseases especially in Middle East, Northern Africa and India [3,4]. Nevertheless, in the existing literature the protective or therapeutic effect of thymoquinone on cigarette smoking-induced vascular damage has not been studied yet. Therefore, we designed this experimental study to determine whether thymoquinone has a protective effect against cigarette smoking-induced vascular damage in rats.

## Methods

### *Experimental animals*

A total of 28 experimental animals were obtained from Bolu Abant Izzet Baysal University (BAIBU) Experimental Animals Research and Practice Center (EARPC). The study was conducted experimentally on 2 to 4 months old male Wistar albino species rats that weighed 200-250 grams. Each animal was kept under constant temperature (24+/-2 °C) and under constant humidity (55+15%) both before and during the study at the EARPC. The rats were allowed to consume standard rat food and water before the study. All experimental applications were approved by Animal Experiments Local Ethics Committee of BAIBU (Decision no: 2019/02/A2).

### *Establishing smoking-induced vascular damage model in animals*

The rats were subjected to cigarette smoke inhalation three times a day with 2 cigarettes given 15 minutes. This application was conducted every day for 2 months.

### *Study groups*

A control group, a group in which cigarette exposure induced vascular damage has occurred, another group with vascular damage and that received Nigella sativa seed extracts and the group consisting of with vascular damage and given dexamethasone were the 4 groups that we had established for our study.

1. Control group (Group 1, n=7): No procedure was performed on the subjects.
2. Only smoking group (Group 2, n=7): For during 2 months every day of each week 3 times a day 2 cigarettes were given through a chamber with a 15 minutes interval between the 2 cigarettes thus ensuring the inhalation of the cigarette smoke current by the subjects.
3. Smoking + Thymoquinone group: (Group 3, n=7): Rats whom were subjected to cigarette smoke inhalation 3 times a day to 2 cigarettes 15 minutes apart for two months thus inducing vascular damage were administered Nigella sativa seed extracts via a feeding tube.

4. Smoking + Dexamethasone group: (Group 4, n=7) : This group was also subjected to same cigarette smoke inhalation and endothelial damage was established; subjects were given 50 micrograms/ mL of dexamethasone through a feeding tube.

***Surgical procedure***

Surgical procedure was performed under general anesthesia with intramuscular 10 mg/kg ksilazin and 90 mg/kg ketamine. After conducting the necessary aseptic practices, for exploration of the thoracic aorta a 2 to 3 cm vertical incision was performed right beneath the cervical part of the rats in the anterior mid-section. While this incision was done in the supine position afterwards the thoracic aorta was dissected free from the surrounding tissue and resected. Resected thoracic aorta tissue samples were kept in 10% formaldehyde solution pending histopathological analysis.

***Histopathological analysis***

Following the resection the thoracic aorta tissue samples were fixed by 10% neutral-buffered formalin. The entire aorta was sampled with 3 mm transvers sections, paraffin embedded and sectioned at a 5 μm thickness according to the standard procedure. The sections were deparaffinized and hydrated gradually, and then examined by hematoxylin and eosin staining. The dyed slices were analyzed under light microscope and were evaluated in terms of degenerative alterations such as presence/absence of atherosclerosis, calcification and fat accumulation as well as inflammation, congestion, dilation and fibrosis on the endothelium. The slices were also evaluated in terms of endothelial continuity and damage, as well as thickness of aortic wall. All histopathological evaluations were carried out by an experienced vascular pathologist.

***Sample size calculation***

Power analysis was used to determine the number of the animals in each group. According to the power analysis; when standard deviation and power were taken as 2 and 0.80 respectively the n value (number of the animals in each group) was found to be 7. Since there were 4 groups, the total number of the subjects was calculated as 28 animals.

***Statistical analysis***

All statistical analyses were performed using the Statistical Package for Social Sciences 20.0 for Windows (SPSS Inc., Chicago, Illinois, USA). In order to determine the differences among the groups; in comparing the continuous variables Kruskal-Wallis test was used while for comparing the categorical variables Pearson Chi-Square test was employed. When a p value was less than 0.05, the differences were considered as statistically significant.

## Results

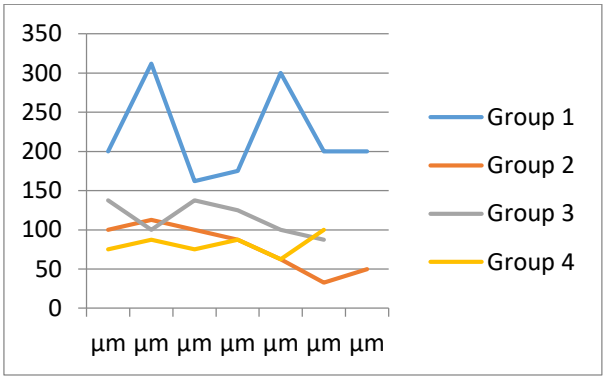
Degenerative alterations on the aortic tissue including atherosclerosis, calcification, fat accumulation, inflammation, congestion, dilation and fibrosis were analyzed in the animals, and no any degenerative alterations on aortic endothelium were observed in all animals in the groups.

Vascular endothelial continuity and damage were analyzed and compared between the groups. In control group (Group 1); partial endothelial damage was observed in only 1 animal, and in remaining 6 animals no endothelial damage was observed. In only smoking group (Group 2); partial endothelial damage was observed in 2 animals, and complete endothelial damage was observed in 4 animals. In only 1 animal no endothelial damage was observed in Group 2. In smoking + Thymoquinone group (Group 3); no

endothelial damage was observed in 2 animals while partial endothelial damage was observed in 4 animals. In smoking + Dexamethasone group (Group 4); no endothelial damage was observed in 3 animals while partial endothelial damage was observed in 3 animals. In both Group 3 and 4, no complete endothelial damage was observed. In terms of vascular endothelial continuity and damage; when Group 1 and 3 were compared the difference was not found to be statistically significant *(p=0.121)*. When Group 1 and 4 were compared in terms of endothelial damage, the difference was not found to be statistically significant *(p=0.273)*. On the other hand, when Group 2 and 3 were compared in terms of endothelial damage, the difference was found to be statistically significant *(p=0.014)*. Similarly, the difference was found to be statistically significant when Group 2 and 4 were compared *(p=0.003)*.

Thickness of aortic walls of animals were measured and compared between the groups. Mean aortic wall thicknesses were 221.2±59.7 µ in control group (Group 1) and 77.8±29.8 µ in only smoking group (Group 2). When Group 1 and 2 were compared, the difference was found to be statistically significant *(p=0.001)*. Mean aortic wall thicknesses in smoking + Thymoquinone group (Group 3) and smoking + Dexamethasone group (Group 4) were 114.5±21.5 µ and 81.25±13.1 µ, respectively (Graphic 1). When Group 3 and 4 were compared, the difference was found to be statistically significant *(p=0.002)*. In addition, when smoking + Thymoquinone group (Group 3) and only smoking group (Group 2) were compared in terms of aortic wall thickness, a significantly mean thicker aortic wall was observed in Group 3 (smoking + Thymoquinone group) *(p=0.001)*.

Microscopic images related to vascular endothelial continuity and damage, and thickness of aortic walls of a subject in the groups are shown in the Figures 1-4.




**Graphic 1.** Aortic wall thickness in all groups.

## Discussion

The main findings of this experimental study were as follows: 1. In animals exposed to only smoking (Group 2), thinness on aortic wall was observed compared to control group (Group 1). 2. In animals exposed to smoking along with thymoquinone administration (Group 3), thickness on aortic wall was observed compared to animals exposed to only smoking (Group 2). This situation reflects that thymoquinone has a protective effect against smoking-induced vascular thinness. 3. In animals exposed to smoking along with thymoquinone administration (Group 3), less vascular endothelial damage was observed than in animals exposed to only smoking (Group 2). This situation reflects that thymoquinone may alleviate smoking-induced endothelial damage. 4. Two-month smoking exposure in rats was no significant effect on degenerative alterations on aortic endothelium, such as atherosclerosis, calcification and fat accumulation.

Cigarette smoking is known to be a preventable risk factor for a variety of clinical conditions including cardiovascular diseases. Previous studies have established the relationship between tobacco consumption and clinical entities such as endothelial dysfunction,

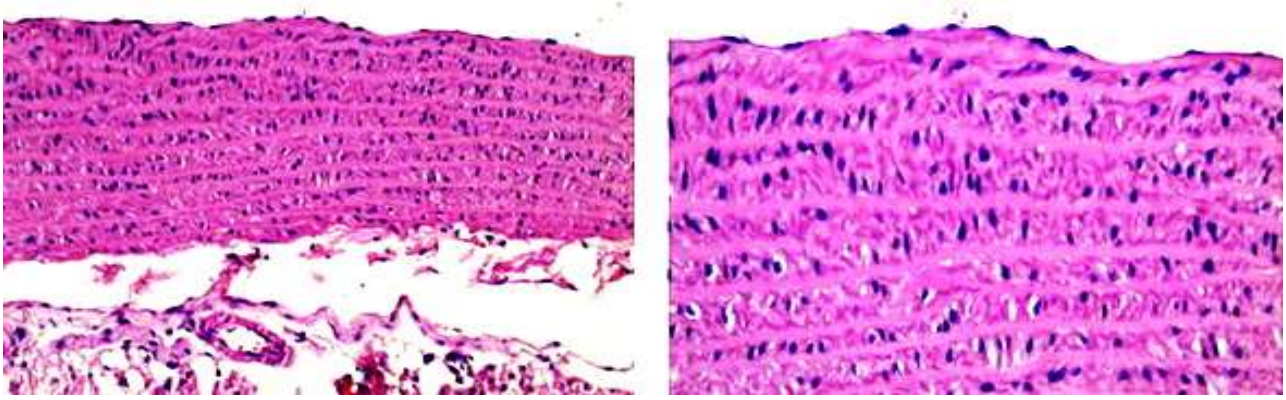


**Figure 1.** Microscopic images of a subject in Group 1 (Hematoxylin & Eosin, magnification x200 and x400).

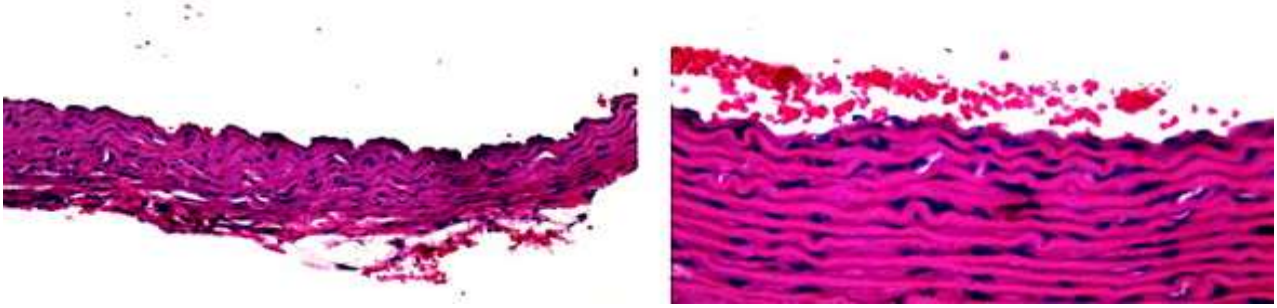


**Figure 2.** Microscopic images of a subject in Group 2 (Hematoxylin & Eosin, magnification x200 and x400).

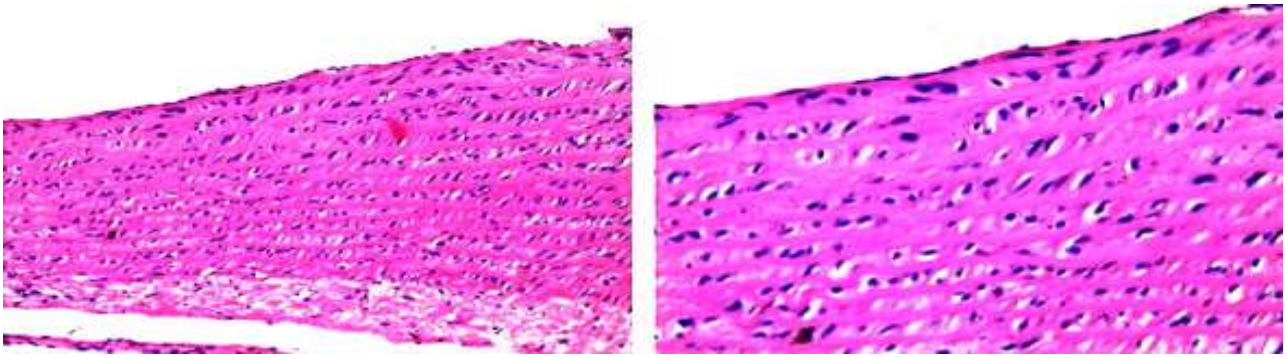


**Figure 3.** Microscopic images of a subject in Group 3 (Hematoxylin & Eosin, magnification x200 and x400).

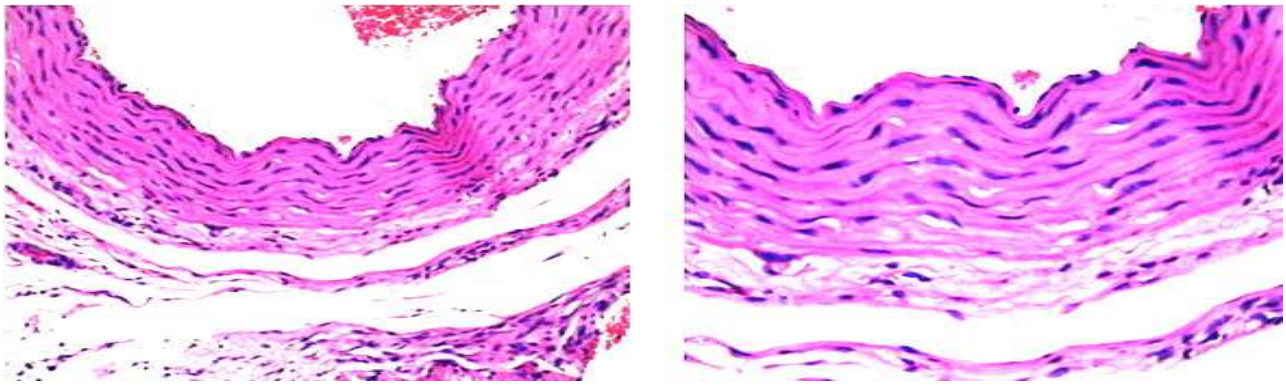


**Figure 4.** Microscopic images of a subject in Group 4 (Hematoxylin & Eosin, magnification x200 and x400).

atherosclerosis and intravascular thrombosis, all which have been shown to be important etiologic factors in the development of hypertension, coronary artery disease and myocardial infarction [1]. Individuals who consume tobacco have lower combined nitrite, nitrate and antioxidant levels in their systemic circulation [5]. Tobacco consumption leads to the disruption L-arginine–nitric oxide synthase (NOS) pathway at the endothelial cellular level which in turn causes decreases in NO (nitric oxide) production, L-arginine transport and expression and activity of NOS [6]. Furthermore, exposure to tobacco or cigarette smoke inhalation causes the increase of the productions of prostaglandin E2 (PGE2), cyclooxygenase-2 (COX-2) and intercellular adhesion molecule-1 (ICAM-1) and these molecular changes establish subclinical inflammatory process atherosclerotic condition in the body [7]. In addition to the aforementioned mechanisms long term exposure to cigarette smoke causes the free oxygen in the plasma to increase which in turn disrupts the oxidant/antioxidant balance which all culminates in increased oxidative stress in the person. Both imbalanced oxidant/antioxidant equilibrium and increased oxidative stress affects the NO activation adversely leading to the increase of endothelial dysfunction and this finally causes the intraluminal blood flow to decrease and end-organ ischemia [8]. In an experimental study in mice that were exposed to cigarette smoke for a long duration (16 weeks), structural features of coronary arteries of mice were affected irreversibly and their vascular elasticity had been reduced [9]. In another experimental study in rats, cigarette smoke exposure was shown to cause the contraction of the basilary artery of rats and these contractions were significantly endothelium-dependent [10]. Drugs and chemical agents that we administer to protect the health and treat the diseases of humans and animals potentially can have adverse effects or even more seriously can cause harm hence lately alternative and/or supplementary medical practices and medications has gained momentum and increasing popularity. Among them herbal origin products are especially widely used [11]. Having very low side effect profile and possessing considerable and interesting roles as nutrition supplement herbal antioxidant usage has become a popular subject nowadays. In a recent study by Kaplan et al. [12], endothelial dysfunction and inflammation that occurs in the thoracic aorta of the mice secondarily after being exposed to cigarette smoke were attempted to neutralize with alpha linolenic acid which is present in flaxseed and soy; as a result alpha-linolenic acid was proven to inhibit the inflammatory mechanisms and prevent the smooth muscle damage caused by cigarette smoking.

Nigella sativa L. that belongs to the Ranunculaceae family is a 20-30 cm plant with flowers and its habitat comprises Southwest Asia, North Africa and Europe. Although climate conditions due to different geographic regions may cause variations in structure, essentially Nigella sativa L. seeds consists of fixed and volatile oils, proteins, amino acids, carbohydrates, alkaloids, tannins, saponins, minerals (calcium, zinc, phosphate) and vitamins (ascorbic acid, thiamine, niacin, pyridoxine and folic acid). The fixed oil contains oleic acid, linoleic acid and arachidonic acid as unsaturated fats while myristic acid, palmitic acid and stearic acid are the saturated fat components. Volatile oils are comprised of d-limonene, nigellone, carvacrol, α and β-pinene, as well as the main pharmacologically active components of thymoquinone, dithymoquinone,

thymohydroquinone and thymol [13]. Nigella sativa L. seeds have been used as a product of natural and traditional medicine for centuries especially in the Middle East, Northern Africa and India and is also being employed for headache, fever, flu, coughing, asthma, bronchitis, eczema, hypertension, diabetes mellitus, obesity, rheumatic diseases and even cancers [14].

Thymoquinone is the main active phenolic hydrophobic substance that is extracted from the volatile oil of Nigella sativa L. seeds. Studies have shown that thymoquinone through different mechanisms has antioxidant, anti-inflammatory, immunomodulatory, anticancer, antimicrobial, hypolipidemic, hypoglycemic, antidiabetic effects, and protective effects on liver, stomach and kidneys and furthermore it is postulated that it can have beneficiary effects on cardiac and respiratory diseases. Also the studies on the toxic profile of thymoquinone have revealed that its toxic effects were seen only with very high doses of thymoquinone [4,14,15]. As a result with its important biological effects and low systemic toxicity the clinical importance of thymoquinone is increasing, thus it can be a promising alternative to the classic therapeutic drugs. However, despite these beneficial effects of thymoquinone, the molecular mechanisms by which these effects are realized have not been elucidated yet.

Considering the potential of antioxidants and anti-inflammatory substances' preventing and curing pathologies characterized with endothelial damage and dysfunction new therapeutic approaches can be devised. In this study our goal was to research the effect of Nigella sativa seeds' active component thymoquinone's effect on the endothelial damage and changes of the rats that were exposed to cigarette smoke. The chosen experimental animal is the most genetically and physiologically compatible with human beings and most suitable species for the study. The substances we will use has been reported to be as antioxidants in previous studies. Research on the preventive effects of these substances on the endothelial damage and dysfunction model created by cigarette smoke could lead to new approaches in the clinical treatments.

The incidence of cigarette related cardiovascular diseases is increasing [1,2]. Researches on oxidant/antioxidant balance regarding the inflammation which is the underlying cause in the etiopathogenesis of occlusive vascular diseases has been frequently carried out. It has been observed that the main goal of the studies is to minimize vascular endothelial damage as much as possible and curing vascular diseases [16-18]. For this purpose, various herbal or chemical compounds have been experimented for antioxidant therapy. In this present study, we used thymoquinone due to its antioxidant and anti-inflammatory properties, and investigated its protective effect on the endothelial damage and dysfunction model established with experimental cigarette smoke inhalation. Our results, whether positive or negative, may be used in further studies. The development of positive results will enable to reveal new approaches in the treatment of oxidative injury in occlusive vascular diseases in the clinical practice.

The main limitation of this study was relatively small sample size. Another important limitation was that not using electron microscopy and using only light microscopy.

## *Conclusions*

To the best of our knowledge, this experimental study is the first one investigating the protective or therapeutic effect of thymoquinone on

cigarette smoking-induced vascular damage. The results of our study revealed that thymoquinone had a protective effect on the vascular endothelium, and alleviated the smoking-induced vascular injury in rats. Nevertheless, further studies are required to achieve high quality evidence.

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***Conflict of Interest:*** *No conflict of interest was declared by the authors.*

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