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Supplementation of Thymoquinone and Carob Together in the Experimental Rat Asthma Model: Oxidative Effect on the Liver Tissue

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ABSTRACT

Asthma, an important public health problem, is a common, potentially serious, medical condition in children, adults and pregnant women. The aim of this study is to investigate the effects of the combined use of thymoquinone and carob on liver tissue oxidative events, following the experimental asthma model. 18 male albino wistar rats were divided into 3 groups as: the control group, the experimental asthma group and treated group (A+TQ+C). In the asthmatic groups, ovalbumin and alum were given intraperitoneally on the 0 and 14th days, and sensitized by inhalation on the 21st, 22nd and 23rd days. In the next 5 days, thymoquinone and carob were given to the group to be treated by intragastric gavage method. In all experimental groups, glutathione (GSH), ascorbic acid (AA), malondialdehyde (MDA) and nitric oxide (NOx) levels were measured spectrophotometrically to evaluate the oxidant-antioxidant status in the liver tissue of rats. While liver tissue GSH and AA levels increased, NOx levels were found to decrease following thymoquinone and carob administration in the treated group (A+TQ+C) when compared other groups (Control and Asthma). However, MDA levels, which are the indicator of lipid peroxidation, were found to be statistically significantly increased in the treated group (A+TQ+C) ($p<0.05$) when compared the other groups (Control and Asthma). As a result, the co-administration of thymoquinone and carob has a regulatory effect that eliminates the possible oxidative effects of asthma, giving hope that it may be a new treatment for children, adults and pregnant women.

Keywords:
Asthma; Thymoquinone; Carob; Liver; Oxidative stress

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INTRODUCTION

Asthma is a common and complex respiratory system disease characterized by high sensitivity of the bronchi, obstruction and inflammation of the airways, affecting a high majority of societies [1-2]. Having different phenotypes of asthma, it affects about 3.5 million people in Turkey [3]. Many factors can cause asthma such as genetic, allergens etc. For this reason, the factors affecting asthma can be divided into two as the ones that cause the formation of asthma and trigger the markers of asthma. Asthma formation, which is the first factor of asthma, generally includes host factors, while environmental factors generally make triggering, which is the second factor [4]. Asthma disease is characterized by specific and nonspecific stimuli, pulmonary eosinophilia, immunoglobulin E (IgE), and increased airway mucus synthesis (AHR) resulting from mucosal synthesis [5]. Inflammation in the airways causes symptoms such as cough, shortness of breath, wheezing and chest tightness in individuals with sensitivity. In cases with asthma, eosinophil, macrophage and lymphocyte levels are quite evident in response to airway inflammation [6]. Mast cells play an important role in asthma while ensuring that they are activated as well as enabling mediators with proinflammatory and constrictive power in the airway [7]. In addition, the effects of interleukins on the inflammatory response and free radical damage are very important in asthma pathogenesis [8]. In addition to the inflammatory response in the airways during the progression of its asthma, characteristic structural changes known as airway remodeling are observed. It is responsible for the occurrence of angiogenesis and microvascular remodeling, as well as many factors in the tissue. Remodeling is associated with airway hypersensitivity and airway narrowing, which is the main physiological feature of the disease.

Bronchial hyper-reactivity (BHR) is related to remodeling and asthma inflammation. One reason for using various asthma medications is to eliminate BHR and thus eliminate remodeling and inflammation [9-10]. Free radicals are very important in asthma pathology. Free radicals with one or more unpaired electrons cause chains of events called oxidative stress that cause tissue damage. In addition, many reagents and radical molecules damage cells and tissues as a result of lipid peroxidation [11-12]. Many free radicals (like NO) can cause to lipid peroxidation. This creates oxidative stress. The cells then use antioxidant defense to get rid of oxidative damage. [13].

The liver is a physiologically important organ that plays a role in the regulation of many metabolic events such as synthesis of biochemical necessary for growth and digestion, glycogen metabolism, hormone synthesis and detoxification of metabolites, control of the endocrine system, regulation of cholesterol and lipid metabolism, storage of vitamins and degradation of xenobiotics [14-15]. In addition to all these, the liver is also responsible for the metabolism of antioxidant defense systems. These defense mechanisms for oxidative stress; it occurs of some vitamins and chemicals such as vitamins A, E, C, β-carotene, reduced glutathione (GSH), and various antioxidant enzymes [16]. In addition, the liver has the highest GSH in the body (20% of the total) [17,18].

The drugs currently produced are effective in regulating remodeling by reducing inflammation. Currently, the most appropriate treatment method for people with asthma is the regular use of inhaled steroids and beta-2 agonists. Despite the use of treatments with side effects, the rates of affecting airway structural changes are lower, although the symptoms of asthma may persist [10, 19-20]. For these reasons, finding a new and natural approach is very important for the treatment of asthma. Many herbs are used as medicines in traditional medicine [21]. Thymoquinone (TQ) is the active ingredient of *Nigella sativa*. In the light of the information obtained, thymoquinone and *Ceratonia silique* (carob) show promise in many diseases, including the respiratory tract, thanks to their anti-inflammatory and protective properties. [22-23].

Thymoquinone, the agent of *Nigella sativa*, has shown anti-inflammatory and antioxidant effects in asthma studies. In addition, while thymoquinone inhibits Cyclooxygenase (COX) and lipoxygenase (LO) inflammation, decreases the ratio of T helper 2 (TH 2) cytokine and eosinophils [24-27]. Economically important carob has beta-carotene, antioxidant, polyphenolic compounds, arachidonic acid, lignin, carbohydrate, fat, various minerals, aspartic acid, glutamic acid, various vitamins, ellagitanins and many more molecules. Carob, which has proven to have anti-allergic, antioxidant, anticancer, antiproliferative effects, is a promising plant in many sectors [28-37].

Side effects of drugs used in many diseases have led people to find more natural approaches. There are no previous reports showing the relationship between carob and asthma. This study is also the first in the absence of a literature showing the oxidative effect of carob and thymoquinone co-administration on the liver in asthmatic models. Our study will help many treatment approaches in this regard and also giving hope that it may be a new treatment for children, adults and pregnant women.

MATERIAL AND METHODS

Experimental Animals and Ethical Approval

Permission for the studies was obtained from the Ethics Committee of Gazi University Experimental Animals (G.U.ET- 16.035) and all the stages up to the collection of tissue samples were carried out in the Gazi University Laboratory Animal Breeding and Experimental Research Center (GUDAM). In the experiments, 18 adult wistar albino male rats (200-250 gr) from GUDAM were used. The animals that were fed ad libitum before and during the experiment were kept in individual cages during the experiment, in an environment of 20 ± 2°C and illuminated in parallel with the daylight cycle. Ovalbumin administration (grade V, ref.A5503-1G, Sigma Aldrich) was administered intraperitoneally to asthmatic groups. The rats are divided into 3 groups, every group having six rats. Animal experiments were carried out in GUDAM and the rest of the experiment was carried out in Gazi University Faculty of Science Physiology-Biochemistry Research Laboratory.

Experimental Design

18 male albino Wistar rats were divided into 3 groups as: the control group, the experimental asthma group (A) and treated group (A+TQ+C). Arrangements were made to have 6 rats in each group and the temperature and humidity of the environment were adjusted to be 20±2°C and %40-60, respectively. All applications carried out throughout the study were carried out in accordance with the ethical guidelines of our university. The animals were fed normally before and during the experiment as ad libitum. The animals were examined in individual cages during the experiment, in the environments illuminated in daylight cycle order. Treated rats received 10mg\kg\day each of TQ and carob once daily for the last 5 days of administration. TQ was dissolved in normal saline (PF %0, 9 isotonic) 10mg\kg using water bath kept at 60°C and the solution was prepared fresh just before gavage administration. The carob pod extract (carob powder

from herbalist) was dissolved in normal saline (PF % 0.9 isotonic) at room temperature and the solution was prepared [38].

Sensitization and Inhalation Exposure

In groups to which asthma models (A and A+TQ+C) were effective sensitized by intraperitoneally (i.p.) of OVA (1mg/ml saline) with alum (1mg/ml saline) (Reagent grade, 239186-25G, Sigma Aldrich) that an adjuvant on days 0 and 14th [39-40]. Subsequently, the study groups were exposed to ovalbumin (5mg/ml saline) inhalation with 30 minutes nebulizer (Handyneb, SN.NGM 769576) on the 21st, 22nd and 23rd days of the study. TQ (2746665G, Sigma Aldrich) and carob pod extract (carob powder from herbalist) solutions were applied by intragastric gavage for 5 days after inhalation. General anesthesia was achieved by weighing the experimental animals on a standard balance and injecting ketamine (Alfamanine 50mg/kg) and xylazine (Alfazyne 5mg/kg) according to their weight. MDA (Malondialdehyde), GSH (Glutathione), AA (Ascorbic Acid) and NO (Nitric oxide) levels were evaluated spectrophotometrically in liver tissue of animals sacrificed 24 hours after application.

Table 1. Sensitization and inhalation exposure in rats

DAYS	0. day	14. day	21. day	22. day	23. day	24. day	25. day	26. day	27. day	28. day
	İ.p. OVA		30 min. OVA inhalation with nebulizer			TQ+C supplementation				

Biochemical Analyses

MDA Determination Method in Tissue

Buege and Aust's method has been applied [41]. Liver tissue was homogenized in 10 volumes (w/v) ice-cold 150 mM KCl for 2 minutes using a glass-Teflon homozygizer at 5000 rpm (Heidolph8F rpmx 1000). The tissue sample was mixed with 2 volumes of cold %15 (w/v) TCA to precipitate the protein. The precipitate formed was centrifuged and some of the supernatant was reacted with an equal amount of %0.67 (w/v) TBA and 1% (w/v) BHT (%95 Ethanol) in a boiling water bath for 10 minutes. Upon cooling, the absorbance was read spectrophotometrically at 532 nm.

GSH Determination Method in Tissue

Elman method was arranged and used for GSH measurement in tissue. Tissue samples were homogenized at 0.15 M cold KCl and 0.5 ml of metaphosphoric acid-EDTA-NaCl mixture was added to the homogenate for deproteinization. After 20 minutes of centrifugation at 4000 rpm at 4oC, 2 ml of DTNB solution (0.4 mg/ml % 1sodium citrate) from 0.5 ml of supernatant was added from 2 ml 0.3M Na2HPO4. All samples were read spectrophotometrically at 412 nm. GSH level in tissue was calculated as μmol per gram tissue [42].

AA Determination Method in Tissue

The method of Roe and Kutherin, edited by Berger, was used. Tissues were homogenized in the thin-cold PCA/EDTA mixture. In the next process, homogenate was centrifuged at 15000g (RCF) for 3 minutes at 4 oC. One tube was placed as standard AA solution, the other as a PCA solution, and samples were prepared in supernatant tubes. The tubes were all incubated individually for 3 hours at 37 °C with the addition of color reagent. The temperature of the samples was brought to 0 °C by adding sulfuric acid to each tube and mixed. The mixture was then left at room temperature for 30 minutes. Samples were read spectrophotometrically at 520nm. AA level in tissue was calculated as μg per ml [40].

NOx Determination Method in Tissue

The NOx level was determined according to the stable end products, nitrite and nitrate. NOx level was determined by Griess reaction in the tissue. In summary, 0.3M NaOH and %10 ZnSO4 were added to 0.5ml sample for deproteinization. The mixture was centrifuged at 3000 g for 20 minutes after the experiment. The supernatants were then used for the Griess experiment. NaNO2 and NO3 solutions (1,10,50,100 μM) were used as standard [43].

Statistical Analysis

All values are expressed as arithmetic mean ± standard error. The values obtained were evaluated using Anova analysis of variance (one-way ANOVA) and Tukey multiple comparison test (SPSS 16.0 for Windows (SPSS, Inc., Chicago, USA). P value of <0.05 was considered statistically significant.

RESULTS

MDA, GSH, AA and NOx levels of our groups are shown in Table 2.

MDA Levels of Groups

Tissue liver MDA levels were shown in Figure1 and Table 2. When the control and asthma groups were compared

Table 2. MDA, GSH, AA and NOx levels in the liver tissues of rats

GROUPS	MDA Level (nmol / gr tissue)	GSH Level (micro-mole/gr tissue)	AA Level (microg-ram /ml)	NOx Level (nmol/gr tissue)
CONT-ROL [n=6]	8,02 ±0,22	11,29±0,26	10,33±0,41	71,63±7,9
AST-HMA [n=6]	9,88±1,10[a]	9,37±0,45*[a]	9,37±0,40*[a]	116,14±0,64*[a]
A+TQ+H [n=6]	13,99±0,84*	11,78±0,70	11,15±0,49[c]	71,77±1,69

*p <0.05 compared to control
[a] p <0.05 compared with A + TQ + C group
[c] p <0.05 compared with the asthma group

with each other, liver tissue MDA levels were found to be increased in the asthma group (Fig.1) (p<0.05). When the asthma group and the treatment group were compared, liver tissue MDA levels were found to be increased in the treatment group (A+TQ+C) (Fig.1) (p<0.05). Lipid peroxidation may have increased as a result of thymoquinone and carob metabolism in the treatment group (A+TQ+C).

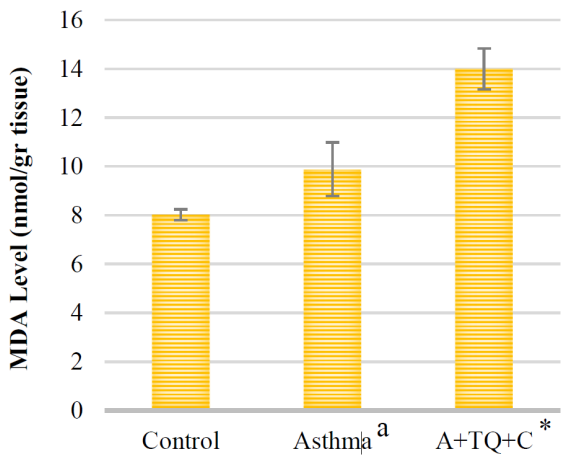

Figure 1. MDA level in the livers of groups.
* p <0.05 compared to control
[a] p <0.05 compared with A+TQ+C group

GSH Levels of Groups

The liver GSH levels were shown in Figure 2 and Table 2. GSH levels were significantly decreased in the asthma group when compared to the other groups. GSH level in the liver tissue of the treated group showed a statistically significant increase compared to the other groups (Fig.2) (p<0.05). GSH production increased in liver tissue with co-administration of TQ+C. This effect may possibly be due to the cumulative effects of the synergistic antioxidant effects of TQ and Carob.

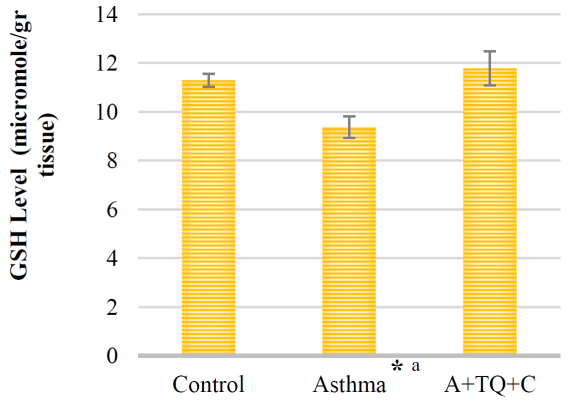

Figure 2. GSH levels in the livers of groups.
* p <0.05 compared to control
[a] p <0.05 compared with A + TQ + C group

Ascorbic Acid (AA) Levels of Groups

Tissue liver AA level were shown in Figure 3 and Table 2. AA levels were significantly decreased in the asthma group when compared to the other groups. AA levels in the liver tissue of the treated group showed a statistically significant increase compared to the other group (Fig.3) (p <0.05). In the treatment group, increased AA levels in liver tissue may have arisen from carob extract.

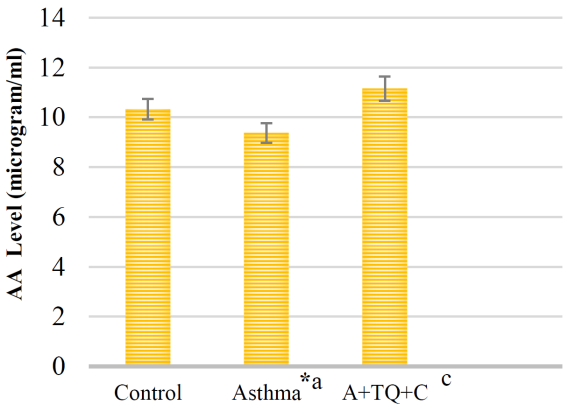

Figure 3. AA levels in the livers of groups.
* p <0.05 compared to control
[a] p <0.05 compared with A + TQ + C group
[c] p <0.05 compared with the asthma group

NOx Levels of Groups

Tissue liver NO levels were shown in Figure 4 and Table 2. NOx levels increased in the asthma group when compared to the other groups due to lipid peroxidation. The NOx level of the treated group showed a statistically significant decrease compared to the asthmatic group (Fig.4) (p <0.05).

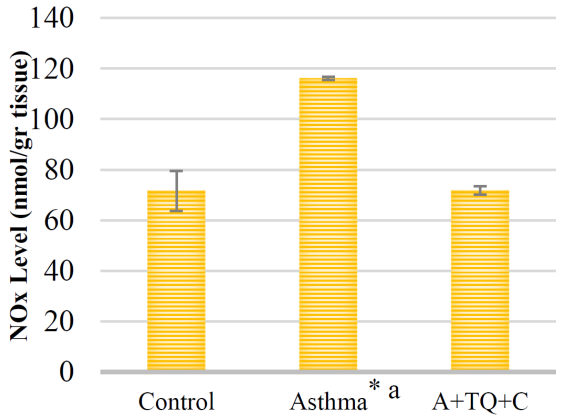

Figure 4. ANOx levels in the livers of groups.
* p <0.05 compared to control
[a] p <0.05 compared with A+TQ+C group

DISCUSSION

Asthma is a disease associated with free radicals and inflammation and induces a state of oxidative stress characterized by an enhancement of lipid peroxidation and depletion of endogenous antioxidant enzyme activities. Corticosteroids are currently used in the treatment of asthma and their side effects are quite common [10,19-20]. For this reason, there has been an excessive interest in herbal sources, that is, non-artificial products, in the treatment or support of various diseases such as asthma [21]. There is no information in the literature that TQ and C are used together in the treatment of asthma except our previous study [38].

Following by OVA exposure, increased lipid peroxidation has been reported in the literature as a result of the penetration of allergen in asthmatic models [44-46]. In our study, the MDA levels in liver tissue increased in asthmatic group. This finding is consistent with other researchers [44-46]. Chechchaki et al. [47] reported that increased levels of MDA were observed in liver and lung tissues with release of large amount of free radicals by activated inflammatory cells in pulmonary alveoli. Additionally, in a study by Oskouei et al, it was shown that TQ lowers the rate of cytochrome C synthesized from mitochondria, and therefore the reduction in cytochrome C causes inhibition of lipid peroxidation by the level of MDA in liver tissue [48]. Houghton et al. demostrated the inhibitory properties of thymoquinone on oxidative stress and lipid peroxidation [49]. Additionally, El-Abhar and coworkers found that thymoquinone protects with free radical-induced stomach damaged rats with its antioxidant properties [50]. On the contrary, in our study, with TQ and C supplementation, tissue MDA levels increased in the treated group. Contrary to expectations, co-administration of TQ and C might have caused an increased lipid peroxidation in liver tissue as a result of the metabolism of these two substances.

Reduced glutathione (GSH) is a tripeptide molecule with an excessive reduction mechanism in the cell [51-52]. GSH levels are considered to be an important marker affecting many inflammatory lung diseases such as asthma. In many studies, the mechanism of action on oxidative stress is determined by GSH [53-55]. In our study, GSH levels increased with co-administration of TQ and C. This effect may possibly be due to the cumulative effects of the synergistic antioxidant effects of TQ and Carob.

In this study, the antioxidant AA, just like GSH, was significantly increased in the treated group (A+TQ+C) when compared to the other groups. The findings showed that our treatment contribute to increase antioxidant capacity to eliminate oxidative stress in asthma due to its high AA content (especially carob). In this study, co-administration of TQ and carob has an antiasthmatic and antioxidant effect by eliminating the harmful effects of oxidative damage. Liver tissue AA levels may also have increased due to the high AA levels in the Carob extract in the treatment group. The findings of this study are consistent with the other researchers [13,56-59].

Even under normal conditions, the liver is an organ with a very high metabolism compared to other organs and tissues. Following the experimental asthma model, decreased nitric oxide levels were found in the liver tissue following the combined use of TQ and Carob. This result shows that these two substances with strong antioxidant effect decreased the nitrosative effect by reducing nitric oxide levels. The results of our liver tissue GSH levels and our NO level results show parallelism and support each other.

NO, which is one of the ROS derivatives, causes lipid peroxidation and oxidative stress occurs when the antioxidant level is less than the produced NO. Therefore, increased antioxidant molecules can eliminate oxidative stress [55]. Mekhoukhe et al. reported that phenolic compounds in carob show antioxidant properties [60]. In our study, the potentiating effects of the antioxidants of these two different compounds may have emerged in order to eliminate the nitrosative effect and increased lipid peroxidation triggered by asthma.

Cysteinyl leukotrienes (CysLTs) are important inflammatory molecules secreted from cells such as macrophages and eosinophils in the airway of asthmatic individuals by activation of the 5-lipoxygenase (5-LO) pathway. In the study of Zhang et al., the importance of CysLTs in asthma is clearly demonstrated. In the light of the obtained data, it was shown that TQ has an anti-asthma effect by inhibiting the LO pathway with CysLTs receptor blockade [61-62]. Some researcher showed that TQ occurs its anti-asthmatic effect by blocking the enzymes of the COX and 5-LO pathway. In

this case, since TXB2 (Tromboksan B2) and LTB4 (Leukotriene B4) will not occur, the antiasthmatic effect demonstrated itself [63-64]. In another study supporting this view, Mansour et al. proved that TQ has an asthma-suppressing effect by blocking 5-LO and LTC4 (Leukotriene C4) synthase enzymes [24]. Based on this information, we can interpret that TQ may also have used the blockade of the 5-LO pathway to eliminate oxidative stress in our study.

In this study, oxidative effect in liver tissue decreased significantly as a result of co-administration of thymoquinone and carob (10mg/kg/ 5 days). We used carob as a treatment ingredient because it has antioxidant and anti-inflammatory effects [36]. It is very important to explain the compounds in the carob plant in order to reveal the reasons for its mechanism of action. According to Pazır et al., Ceratonia siliqua plant contains many phenolic compounds, minerals and also D-pinitol, which has anticarcinogenic effects. Because carob contains all these compounds, it is good for obesity, cardiovascular and many other diseases [65]. The antiasthmatic effect of carob has not been studied before. In order to see the antiasthmatic effect of carob, a stand-alone experimental setup may also be arranged. It has been shown that TQ alone cannot produce an antiasthmatic effect at low doses [66]. According to these data, the amount of TQ we used in the study alone is not enough for the antiasthmatic effect. Therefore, it can be said that the significant difference in the results of the treated group is due to the antiasthmatic mechanism that emerged as a result of the combined administration of TQ and carob.

CONCLUSION

This study, following the experimental asthma model, showed the protective properties of two powerful antioxidants (TQ and Carob) in liver tissue against inflammatory events in asthma, thus revealing the positive effects of a mixture that they can use with peace of mind for women, children, pregnant women and individuals with low immunity. It will also pave the way for more multifaceted research on asthma.

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CONFLICT OF INTEREST

Ozge Akyazi confirms, on behalf of all authors, that the information provided is accurate.

AUTHOR CONTRIBUTION

Ozge Akyazi and Sule Coskun Cevher designed the study. OA performed all experiments. OA and SCC analysed the data. OA and SCC wrote the paper.

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