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Protective effect of pretreatment with thymoquinone against Aflatoxin B_1 induced liver toxicity in mice

[1]Nili-Ahmadabadi A., [1,2]Tavakoli F., [3]Hasanzadeh GR., [1]Rahimi HR., [*1,4]Sabzevari O.

[1]Department of Toxicology & Pharmacology, [2] Basic and Clinical Toxicology and Poisoning Research Centre, Faculty of Pharmacy, [3]Department of Histopathology, Faculty of Medicine, [4]Drug Design and Development Research Center, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran.

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ABSTRACT

Background and the purpose of the study: Thymoquinone (TQ) is one of the active components of *Nigella sativa*. The plant has been used in herbal medicine for treatment of many diseases including liver complications. The present study aimed to investigate protective effects of TQ on Aflatoxin B_1 (AFB_1) induced liver toxicity in mice.

Methods: Animals were divided into six groups and treated intraperitoneally. Group 1 (blank) served as vehicle, group 2 (positive control) received AFB_1, Group 3 was treated with 9 mg/kg of TQ, Groups 4, 5 and 6 were treated with 4.5, 9 and 18 mg/kg of TQ, respectively. After three consecutive days, except for groups 1 and 3, animals were administered with a single dose of AFB_1 (2 mg/kg). All the animals were killed 24 hrs following the AFB_1 administration under ether anesthesia. Biochemical parameters including AST, ALT and ALP in serum samples and glutathione (GSH) and malondialdehyde (MDA) contents in liver homogenates were determined. Liver sections were collected for histopathological examination.

Results: Findings of this study showed that AST, ALT, ALP and MDA levels were significantly lower in the TQ treated animals as compared to AFB_1 group (group 2). Furthermore, TQ was able to recover glutathione content (GSH) of liver tissue. The best response, however, was observed with the dose of 9 mg/kg. Liver sections of AFB_1 intoxicated mice showed inflammation, necrosis, hyperplasia of kupffer and infiltration of mononuclear cells, dilation of sinusoids and disruption of hepatocytes, while treatment with TQ helped to normalize liver architecture in accordance to biochemical findings.

Conclusion: Taken collectively, TQ has a protective role with optimum dose of 9 mg/kg in AFB_1 hepatotoxicity.

Keywords: *Nigella sativa*, Glutathione, Malondialdehyde, Hepatocytes, Biochemical findings.

INTRODUCTION

The World health organization (WHO) estimates that 80% of the population in some Asian and African countries are mostly dependent to traditional medicine for their health cares (1). In other words, about 4 billion people in the world rely on plants as source of drugs (2). Among these herbal medicines, *Nigella sativa* is an encouraging medical plant reported to have potent antioxidant effects, and has been used widely throughout the world. The biological activity of *N. sativa* is related to it volatile oil compounds. Thymoquinone, 2-isopropyl-5-methyl-1, 4-benzoquinone, is the major active component of *N. sativa* and constitutes about 30% of its seed extract (3). It has been demonstrated that the *N. sativa* and its chemical components produce a variety of pharmacological actions such as anti-inflammatory, anticancer and antioxidant properties and many of these effects are due to TQ (4, 5). The beneficial medical effects of *N. sativa* and TQ have been related to their radical scavengering activities (6).

AFB_1 is a potent hepatotoxic and hepatocarcinogenic compound produced by the fungus, *Aspergillus flavus* (7). The toxicity and carcinogenicity of AFB_1 is thought to be directly linked to its bio-activation, resulting in a highly reactive AFB_1 8, 9-epoxide (8), which is responsible for binding to cellular macromolecules such as RNA, DNA and other protein constituents. AFB_1 mediated cell damage may be due to in vitro and in vivo free radicals release (9) and these radicals initiate lipid peroxidation process

Correspondence: omid@tums.ac.ir

and antioxidant depletion.
Since oxidative stress processes play an important role in AFB_1-induced hepatotoxicity and TQ possess strong anti-oxidative as well as anti-inflammatory, it was reasonable to hypothesize that the TQ could be protective against AFB_1-induced hepatotoxicity. Accordingly, this study was undertaken to investigate whether TQ protects against AFB_1-induced acute hepatotoxicity in mice and if so, whether the protection is dose-dependent.

MATERIAL AND METHODS

Chemicals
Trichloroacetic acid (TCA), 2-thiobarbituric acid (TBA), *n*-butanol were purchased from Merck Chemical Company (Darmstadt, Germany). AFB_1 and TQ were provided from Sigma-Aldrich Chemical Company (St. Louis, MO, USA).

Animals
Male albino mice (22-27 g) were obtained from animal house of Faculty of Pharmacy, Tehran University of Medical Science and quarantined for one week prior to use. The animals were kept under controlled environmental conditions of temperature (25°C), relative humidity of 50-55%, and 12 hrs light/dark cycle. Mice had free access to stock laboratory diet (Champus dam) and water.

Experimental protocol and groups
Following 1 week of acclimatization, the animals were randomly divided into six groups, six in each. The animals were treated for three consecutive days by intraperitoneal (i.p) injection as follows: Group 1 (Control group) received normal saline solution. Group 2 (positive control) received 2mg/kg of AFB_1. Group 3 received 9 mg/kg of TQ, and groups 4, 5 and 6 were administrated AFB_1 following pretreatment with 4.5, 9 and 18 mg/kg of TQ, respectively. After the treatment period was over, except for groups 1 and 3, animals were treated with a single dose of AFB_1 (2 mg/kg). All the animals were killed 24 hrs following AFB_1 treatment under ether anesthesia.

Determination of ALT, ALP and AST activities
Blood samples were obtained by cardiac puncture, and serum collected for biochemical assays. Activities ALT, ALP and AST were spectrometrically determined using kinetic method employing Ellitech diagnostic kits (Sees, France) according to the manufacturer protocols.

Liver samples
Liver samples were also removed by transverse abdominal incision and perfused with cold 0.9% NaCl, homogenized with three volumes of a solution containing 140mM potassium phosphate buffer (pH 7.0) and centrifuged at 900 *g* for 15 min at 4°C. The supernatant was used as homogenate to assay MDA and GSH concentration. Before the homogenate process, a portion of the liver was fixed in 10% formalin for histopathological studies (10).

Measurement of lipid peroxidation
Lipid peroxidation on liver samples was determined by the reaction of TBA with MDA, the end product of lipid peroxidations (11). Briefly, liver homogenate samples were mixed with trichloroacetic acid (20%) and the precipitate was dispersed into H_2SO_4 (0.05 M). TBA (0.2% in 2 M sodium sulfate) was added and heated for 30 min in boiling water bath. Lipid peroxidation adducts were extracted by *n*-butanol and absorbance was measured at 532nm. Results were expressed as nmol MDA/g tissue.

Measurement of GSH
GSH were determined by the standard methods (12). Results were expressed as µmol GSH/g tissue.

Statistical analyses
The data were expressed as means±S.E. and compared using one way analysis of variance (ANOVA). Comparisons among groups were made according to Tukey-Kramer's multiple comparisons test. The significance level was tested at $p<0.05$.

RESULTS

Effect of TQ on AST, ALT and ALP serum levels
Administration of AFB1 significantly increased serum levels of AST, ALT and ALP (Table 1). TQ pretreatment was able to reduce the increased levels of these enzymes at the employed doses of 4.5, 9 and 18 mg/kg. The best response was obtained by the dose of 9 mg/kg.

Effect of TQ on MDA production in liver
MDA formation, an indicator of lipid peroxidation, was expressed as nmol/g tissue. MDA production was increased significantly by administrating of AFB_1 in comparison to control. However, TQ treatment at doses of 4.5, 9 and 18 mg/kg significantly prevented MDA production (Table 2). The best response was obtained by the dose of 9 mg/kg.

Effect of TQ on GSH content of liver
Table 2 demonstrates the total GSH content of the liver homogenates. Administration of AFB_1 significantly reduced GSH content of liver. Pre-treatment with TQ (4.5, 9 and 18 mg/kg) was found to restore significantly hepatic supply of GSH compared to the AFB_1 group. The best response was observed at the dose of 9 mg/kg. Treatment with only TQ (9 mg/kg) enhanced hepatic GSH content slightly (Table 2).

Histopathological changes
Table 3 shows histopathological changes in mice

Table 1. Protective effect of thymoquinone (TQ) on serum enzyme levels in mice. Statistical analysis used one-way ANOVA with Tukey's test. Values are expressed as means±SE, n=6 for each group. *Significantly different from AFB_1 group ($P < 0.05$); **Significantly different from AFB_1 group ($P < 0.01$); ***Significantly different from AFB_1 group ($P < 0.001$); #Significantly different from control group ($P < 0.001$).

Groups	ALT (U/L)	AST (U/L)	ALP (U/L)
Control	31.2 ± 5.2	93.7 ± 6.2	69.3 ± 6.1
TQ (9 mg/kg)	27.5 ± 5.5	90.2 ± 5.6	63.2 ± 6.0
AFB_1 (2 mg/kg)	99.8 ± 11.9#	174.3 ± 11.5#	226.1 ± 15.6#
TQ (4.5 mg/kg) + AFB_1 (2 mg/kg)	77.4 ± 5.9*	145.8 ± 7.6*	190.6 ± 9.5*
TQ (9 mg/kg) + AFB_1 (2 mg/kg)	51.4 ± 4.8***	130.3 ± 13.2**	154.3 ± 11.9***
TQ (18 mg/kg) + AFB_1 (2 mg/kg)	80.4 ± 5.8*	147.6 ± 11.1*	195.4 ± 12.3*

ALT: Alanine aminotransferase
AST: Aspartate aminotransferase
ALP: Alkaline phosphatase

Table 2. Histopathological changes of liver in experimental groups. Thymoquinone (TQ) was used in doses of 4.5, 9 and 18 mg/kg. Histopathological changes including inflammation (Inflam), necrosis (Nec), hyperplasia of kupffer cells (HKC), infiltration of mononuclear cells (IMNC), dilation of sinusoids (DS) and disruption of hepatocytes (DH) were studied in comparison to control. Changes: +Moderate, ++Severe, +++Very severe.

Groups	Inflam	Nec	HKC	IMNC	DS	DH
Control	–	–	–	–	–	–
TQ (9 mg/kg)	–	–	–	–	–	–
AFB_1 (2 mg/kg)	+++	+++	+++	+++	+++	+++
TQ (4.5 mg/kg) + AFB_1 (2 mg/kg)	++	+	++	++	+	+
TQ (9 mg/kg) + AFB_1 (2 mg/kg)	+	–	+	+	+	–
TQ (18 mg/kg) + AFB_1 (2 mg/kg)	++	+	++	++	++	++

liver following AFB_1 administration and with TQ pretreatment. By administration of only AFB_1, some histopathological changes in liver including inflammation, necrosis, disruptor of hepatocytes, hyperplasia of kupffer cells, infiltration of mononuclear cells and increased diameter of hepatocytes were observed. TQ prevented the histopathological changes of AFB_1 and decreased the number of inflammatory cells. The best response was provided by the dose of 9 mg/kg of TQ. Diameter of hepatocyte as a semi-quantitative indicator of damage to the hepatocytes (13) was reduced to 36% by TQ (9 mg/kg) in comparison to AFB_1 group (Table 4).

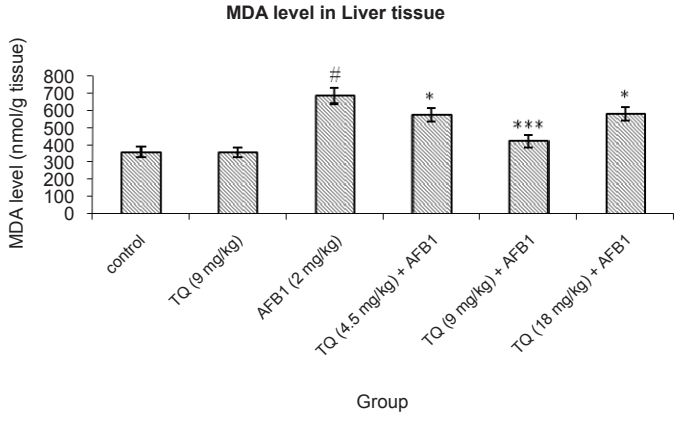
DISCUSION

AFB_1 can induce oxidative stress which may lead to liver injury (14). Protective activity of TQ was evaluated in this study against liver toxicity induced by AFB_1 in mice.

The serum levels of hepatic enzymes primarily reflect the degree of liver damage and have been commonly used as a diagnostic marker for hepatotoxicity (15, 16). As shown in Table 1, AFB_1 (2 mg/kg) significantly elevated ALP, AST and ALT which is in agreement with results of other studies (7, 17). This finding is related well with histopathological examination in which necrosis and degradation, disturbed radiated hepatocytes, hyperplasia of kupffer cells, infiltration of mononuclear cells, inflammation and increase in hepatocytes diameter in liver sections were observed. TQ prevented the histopathological changes by AFB_1 and decreased the number of inflammatory cells in a good relation to the biochemical findings.

Research has shown that TQ, an active principle of *Nigella sativa*, has hepatoprotective activity against tert-butyl hydroperoxide (TBHP) toxicity by preventing depletion of GSH and decreasing liver enzymes leakage including ALT and AST leakage in isolated rat hepatocytes (18). In this study, the best response in reducing serum levels of hepatic enzymes including AST, ALT and ALP, was observed at the dose of 9 mg/kg. Higher doses of TQ (18 mg/kg), however, provided lesser hepatoprotection which might be due to the dose which is close to the chemical toxic dose (estimated LD50 104.7 mg/kg; 89.7-119.7, 95% confidence interval) for mice (19). In a preliminary study, it was found that the dose of TQ toxicity is 50-100 mg/kg, while a study with similar doses (50-100 mg/kg) in Saudi-Arabia had shown hepatoprotection. It may be concluded that TQ toxicity or TQ resistance have a role in the event. The protection monitored by the lower dose of TQ (4.5 mg/kg) was also less significant; therefore, it

1a



1b

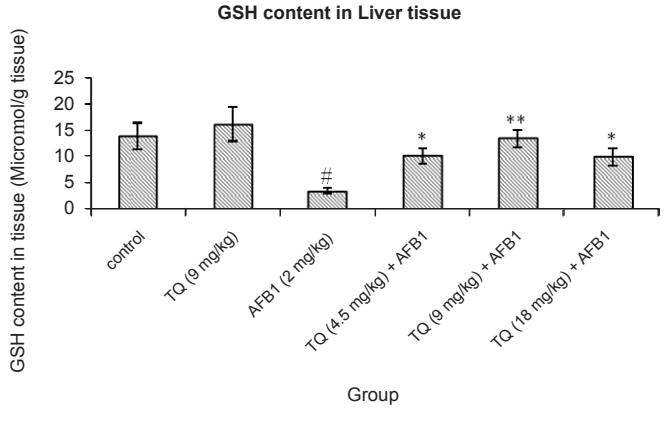


Figure 1 (a & b). Effects of thymoquinone (TQ) on malondialdehyde (MDA) and gluthatione content (GSH) of mice liver. Statistical analysis used one-way ANOVA with Tukey's test. Values are expressed as means±SE, n=6 for each group. *Significantly different from AFB_1 group ($P < 0.05$); **Significantly different from AFB_1 group ($P < 0.01$); ***Significantly different from AFB_1 group ($P < 0.001$); #Significantly different from control group ($P < 0.001$).

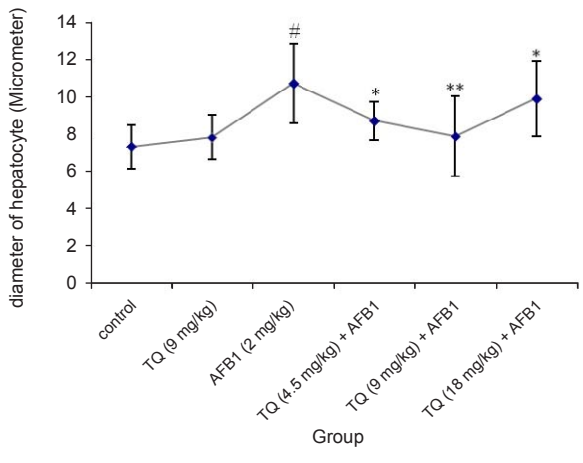


Figure 2. Effects of thymoquinone (TQ) on diameter of hepatocyte Statistical analysis used one-way ANOVA with Tukey's test All data are presented as mean±SE, n=6 for each group. *Significantly different from AFB_1 group ($P < 0.05$); **Significantly different from AFB_1 group ($P < 0.01$).

seems that optimum protective dose of TQ in mice liver toxicity induced by AFB_1 is about 9 mg/kg.

Oxidative stress is one of the main indications of tissue damages and has been found to play an important role in the toxicity and carcinogenesis of many toxicants (20, 21). GSH, a key antioxidant, is an important constituent of intracellular protective mechanisms against various noxious stimuli, including oxidative stress (7). In this study, hepatic level of MDA as lipid peroxidation index was reduced by TQ pre-treatment while GSH content was considerably restored for toxicity prevention. In this regard, maximum protection of liver cells against lipid peroxidation and cellular damage was obtained at the dose of 9 mg/kg. These results further confirm the antioxidant properties of TQ in decreasing hepatic toxicity induced by AFB_1 or other toxins including tert-butyl hydroperoxide (18).

In conclusion, TQ is effective in protection of mice against AFB_1-induced hepatotoxicity possibly via increased resistance to oxidative stress as well as the ability to reduce lipid peroxidation at the optimum dose of 9 mg/kg.

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