Neuroprotective effect of thymoquinone in acrylamide-induced neurotoxicity in Wistar rats

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ARTICLEINFO	ABSTRACT
	Objective(s): Acrylamide (ACR) has broad applications in different industries. It also forms in food during heating process. Oxidative stress has a critical role in ACR-induced neurotoxicity in both *in vitro* and *in vivo* models; therefore, the aim of the current study was the evaluation of effects of thymoquinone, the main constituent of volatile oil from *Nigella sativa* seeds in ACR-induced neurotoxicity. ***Materials and Methods:*** Male Wistar rats were treated with ACR (50 mg/kg IP) alone or with thymoquinone (TQ) (2.5, 5, 10 mg/kg IP) for 11 days. Two protocols were used in this study, A: in this one TQ and ACR were used simultaneously, B: Administration of TQ was started 1 week before ACR treatment and continued during exposure to ACR. At the end of the treatment, behavioral index (gait score) was examined for rats. After that, rats were sacrificed and molondialdehyde (MDA) as a marker of lipid peroxidation and glutathione (GSH) content were determined in cerebral cortex. ***Results:*** Exposure to ACR led to severe gait abnormalities and treatment with TQ significantly decreased abnormalities. Level of MDA was elevated in cerebral cortex after exposure to ACR while TQ treatment significantly and in a dose-dependent manner reduced lipid peroxidation. Results clearly showed that there is no significant difference between two protocols of administration of TQ. ***Conclusion:*** It suggests the neuroprotective effect of TQ in this model in part, may be because of due the antioxidant activity of this natural compound.

Introduction

Acrylamide is a white and water soluble monomer which has different applications in the industrial processes. In addition to occupational sources, diet has been considered as another important rout of ACR exposure, because ACR is found in carbohydrate- rich foods which are cooked in high temperature (1, 2). ACR demonstrated neurotoxic effects in animals and human populations based on different pharma-cological and epidemiological studies (1, 3). Both the peripheral and central nervous systems of animals have been affected after exposure to ACR, led to produce symptoms such as ataxia, skeletal muscle weakness, hindlimb foot splay, ataxia, weak legs, loss of sensation and diminished reflexes (1, 3). Inhibition of neurotransmission by disrupting presynaptic nitric oxide (NO) signaling, nerve-terminal degeneration (3), axonal degeneration (3), increment of lipid peroxidation (4, 5), reduction of antioxidant capacity of nervous system (4) and induction of apoptosis (6, 7) signaling are various mechanisms which are mediated ACR neurotoxicity. Because of importance of ACR toxicity, researchers have focused to find protective compounds which can reduce neurotoxic effects of ACR through disruption of its mechanistic pathway using *in vitro* and *in vivo* models (7-9). Previously we reported effect of some well-known antioxidants such as crocin (7), linalool (10) and chrysin (11) against ACR-induced neurotoxicity.

Nigella sativa L., commonly known as black cumin or black seed, is used in folk medicine as a herbal remedy for many disease and condition such as, inflammation, hypertension, asthma, bronchitis, diabetes, headache, eczema and gastrointestinal disturbances (12). Thymoquinone (TQ) is the main constituent of volatile oil from *N. sativa* seeds which has exhibited different properties in modern pharmacology including anti-inflammatory (13), anticonvulsant (14), antitussive (15), anti-tumor (13, 16) and antioxidant activity (13).

Administration of TQ attenuated hepatotoxicity induced by CCl₄ possibly through inhibition of lipid peroxidation (17). In another study, TQ could reduce hepatic injury in New Zealand rabbits which fed a high-cholesterol diet. It normalized the enhanced hepatic tissue MDA and reduced the protein carbonyl content levels (18). Evidence suggested neuroprotection effects of this well-documented antioxidant. Following cerebral ischemia-reperfusion injury (IRI) in rat hippocampus, level of TBA reactive substances was increased while TQ reduced the brain damage (19). TQ was effective in protecting rats against hippocampus injury induced by transient forebrain ischemia. The recommended mechanisms were elevation of GSH content, superoxide dismutase (SOD) and catalase (CAT) activity (20). Pretreatment of primary cultured cerebellar granule neurons with TQ significantly reduced $A\beta_{1-40}$-induced apoptosis via both extrinsic and intrinsic caspase pathways (21). The intracerebroventricular administration of TQ inhibited the epileptic seizures induced by pentylenetetrazol (PTZ) in rats, probably through an opioid receptor-mediated increase in gamma-Aminobutyric acid (GABA)ergic tone (22). A marked increase in number of apoptotic neurons was observed after chronic toluene exposure in rats. Interestingly, administration of TQ caused morphologic improvement on neurodegeneration in the frontal cortex tissues (23).

Considering the beneficial neuroprotective and antioxidant effects of TQ which mentioned above, the current research was designed to investigate the possible protective effects of this agent in ACR-induced neurotoxicity in Wistar rats.

Materials and Methods
Materials
ACR was purchased from Merck (Germany). TQ and malondialdehyde tetrabutylammonium were prepared from Sigma (Germany) and Sigma (Switzerland). TQ was dissolved in Tween 80 (0.8% v/v) and saline (NaCl 0.9%) solution.

Animals
Male Wistar rats, 230-260 g were housed in colony rooms with 12/12 hr light/dark cycle at 21±2°C and had free access to food and water. All animal experiments were carried out in accordance with Mashhad University of Medical Sciences, Ethical committee Acts.

Experimental protocol
For induction of neurotoxicity in Wistar rats, ACR at daily dose of 50 mg/kg/day intraperitoneal (IP) for 11 days was used. This daily dose-rate and the route of administration have been well characterized with respect to neurological deficits. In the current

study the rats were divided at random into 10 groups (n=6 in each group) and treatment was done follows:
1) Control, Normal saline
2) ACR, 50 mg/kg IP for 11 days
3) ACR, 50 mg/kg IP for 11 days + TQ 2.5 mg/kg
4) ACR, 50 mg/kg IP for 11 days + TQ 5 mg/ kg
5) ACR, 50 mg/kg IP for 11 days + TQ 10 mg/kg
   In groups 3-5, administration of TQ (IP) was started with ACR for 11 days (Protocol A).
6) ACR, 50 mg/kg IP for 11days + vitamin E 200 mg/ kg (IP, three times per week) (10, 11)
7) TQ 10 mg/kg
8) ACR, 50 mg/kg IP for 11 days + TQ 2.5 mg/kg 1 week before administration of ACR and continued during treatment with ACR
9) ACR, 50 mg/kg IP for 11 days + TQ 5 mg/kg 1 week before administration of ACR and continued during treatment with ACR
10) ACR, 50 mg/kg IP for 11 days + TQ 10 mg/kg 1 week before administration of ACR and continued during treatment with ACR
    In groups 8-10, administration of TQ (IP) was started 1 week before administration of ACR and continued during treatment with ACR (Protocol B).

The behavioral index (gait scores) examination
After finishing animal treatment, the gait scores were examined according to the methods which described previously by LoPachin *et al* (3). Rats were placed in a clear Plexiglass box and were observed for 3 min and a gait score, from 1 to 4, was assigned; where 1 = a normal, unaffected gait; 2 = a slightly affected gait (foot splay, slight hindlimb weakness and spread); 3 = a moderately affected gait (foot splay, moderate hindlimb weakness, moderate limb spread during ambulation,); and 4 = a severely affected gait (foot splay, severe hindlimb weakness, dragging hindlimbs, inability to rear).

Tissue sampling
After determination of gait score, rats were sacrificed and the cerebral cortex was dissected, then samples were snap-frozen in liquid nitrogen and stored at −80°C until use.

Lipid peroxidation (level of MDA) assay
The quantitative determination of lipid peroxidation was done by determining the concentration of the malondialdehyde (MDA) levels, as marker of lipid peroxidation, in cerebral cortex (10, 24). MDA reacts with thiobarbituric acid (TBA) as a thiobarbituric acid reactive substance (TBARS) to make a pink colored complex which has maximum absorbance at 532 nm.

In this method, 3 ml phosphoric acid (1%) and 1 ml TBA (0.6%) were added to brain tissue homogenate 10% in KCl, and then the mixtures were heated for 45 min in a boiling water bath. After

cooling the mixture, 4 ml of n-butanol was added and vortex-mixed for 1 min followed by centrifugation at 3000 g for 10 min. Then, the organic layers were removed and transferred to a fresh tube and absorbance was recorded at 532 nm (25). A calibration curve was plotted using malondialdehyde tetrabutylammonium. MDA levels were expressed as nmol/g tissue.
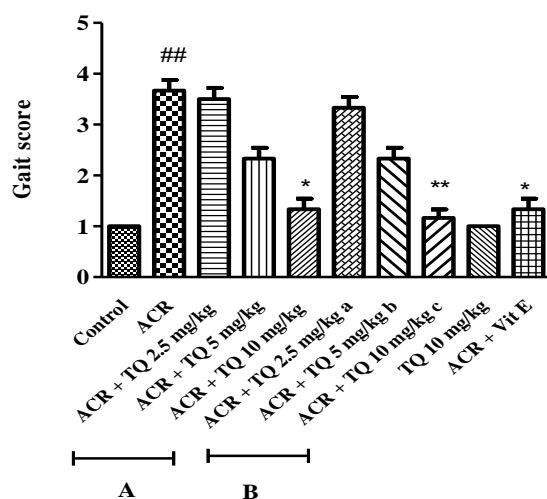
Statistical analysis

Results are expressed as mean ± SD. Statistical analyses for body weight changes and lipid peroxidation were performed with ANOVA followed by Tukey–Kramer test to compare the differences between means. Differences were considered statistically significant when *P*<0.05. Statistical analysis for the gait abnormalities were done with nonparametric test Kruskal-Wallis followed by Dunn's Multiple Comparison Test.

Results
Effect of TQ on the gait abnormalities induced with ACR

As shown in Figure 1, exposure to ACR (50 mg/kg, IP) for 11 days induced progressive gait abnormalities in rats. Administration of TQ dose-dependently improved severe gait abnormalities in animals as compared to ACR- treated group. Results exhibited that there is no difference in the efficacy of TQ when used simultaneously with ACR or 1 week before it. Exposure to TQ (10 mg/kg) alone didn't change gait score in animals as compared to control (*P*>0.05).

Table 1. Effect of thymoquinone on body weight changes in rats during the treatment with Acrylamide

Group	% Body weight change
Control	9.5 ± 0.5
ACR (50 mg/kg)	-9.65 ± 0.7###
A	
TQ (2.5 mg/kg) + ACR	-3.06 ± 0.2***
TQ (5 mg/kg) + ACR	-0.8 ± 0.11***
TQ (10 mg/kg) + ACR	1.25 ± 0.14***
B	
TQ (2.5 mg/kg) + ACR	-3.19 ± 0.13***
TQ (5 mg/kg) + ACR	-0.5 ± 0.05***
TQ (10 mg/kg) + ACR	1.75 ± 0.1***
TQ (10 mg/kg)	1.25 ± 0.14
Vit E + ACR	4.15 ± 0.2***

Data are expressed as the mean ± SD, (n=6). A: TQ was used simultaneously with ACR (50 mg/kg). B: TQ was used 1 week before starting of ACR administration (50 mg/kg) and continued during treatment with ACR. ###*P*<0.001 vs control and ***P*<0.001 vs ACR treated animals
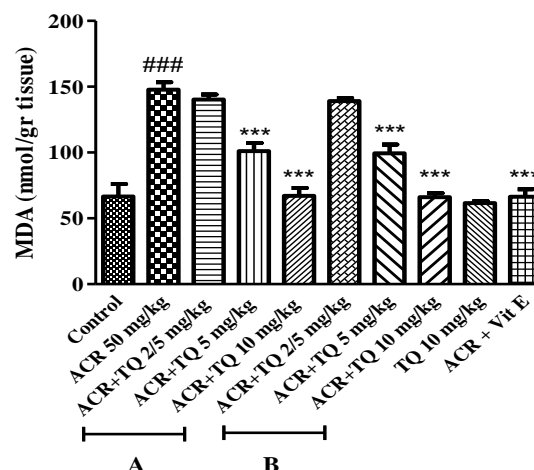
Effect of TQ on the body weight changes induced with ACR

Administration of ACR caused 9.6% decrease in body weight as compared to control group (*P*<0.001). But administration of TQ significantly inhibited body weight loss. As shown in Table 1, TQ (10 mg/kg) increased body weight in comparison to ACR treated animals (*P*<0.001). No significant differences were found between protocols A and B.

Effect of TQ on brain lipid peroxidation following exposure to ACR

Lipid peroxidation in cerebral cortex tissue was determined using determination of MDA content.

As shown in Figure 2, the level of MDA significantly increased in ACR groups (147.83±5.56 nmol/g tissue) when compared to control animals (66.66±9.48 nmol/g tissue) (*P*<0.001).

Figure 1. Effects of thymoquinone on Behavioral Index (gait scores) in rats during the treatment with ACR (50 mg/ kg, IP) for 11 days. Data are expressed as the mean ± SD, (n=6). ##*P*<0.01 vs control, *P*< 0.05 and **P*<0.01 vs Acrylamide treated animals. A: TQ was used simultaneously with ACR (50 mg/kg). B: TQ was used 1 week before starting of ACR administration (50 mg/kg) and continued during treatment with Acrylamide

Figure 2. Effect of thymoquinone on brain lipid peroxidation induced with Acrylamide in cerebral cortex. Data are expressed as the mean±SD, (n=6). ### *P*<0.001vs. control, ***P*<0.001 vs. ACR treated animals. A: TQ was used simultaneously with ACR (50 mg/kg). B: TQ was used 1 week before starting of ACR administration (50 mg/kg) and continued during treatment with Acrylamide

Treatment with TQ at the doses of 5 and 10 mg/kg significantly reduced the level of MDA in cerebral cortex (*P*<0.001). There was not any difference between administration of TQ simultaneously with ACR and usage of it 1 week before ACR. Treatment with TQ (10 mg/kg) alone had no effect on the MDA level (*P*>0.05 vs control group).

Discussion

The results of the present study indicated exposure to ACR (50 mg/kg IP for 11 days) induced gait abnormalities in Wistar rats. Administration of TQ significantly reduced gait scores in animals when received it simultaneously or 1 week before treatment with ACR. Level of MDA increased in cerebral cortex after exposure to ACR, while TQ treatment significantly decreased lipid peroxidation. Animal's body weight change was another marker that dramatically reduced following exposure to ACR, but administration of TQ could decrease the weight loss.

ACR is a well-known neurotoxic agent which damages both central and peripheral nervous system. Ataxia, skeletal muscle weakness and body weight loss have been reported following exposure to ACR in experimental animal models (3, 4). According to our results, administration of ACR (50 mg/kg, IP for 11 days) markedly induced gait abnormalities in animals, while TQ treatment (10 mg/kg) significantly improved animal's abnormalities.

9.6 % weight loss was observed in ACR treated animals, but TQ in a dose-dependent manner could inhibit this significant reduction.

Induction of oxidative stress following treatment with ACR has been mentioned as one of important mechanisms of ACR –induced toxicity in different *in vivo* and *in vitro* models (4, 7). Depletion of GSH content, enhancement of lipid peroxidation, protein carbonyl content and hydroxyl radicals have been reported in ACR toxicity (24). Therefore, researchers tried to find neuroprotective agents in ACR-induced neurotoxicity with focus on inhibition of oxidative stress pathway. Recently, we reported neuroprotective effects of linalool against ACR-induced neurotoxicity through enhancement of GSH content and reduction of lipid peroxidation in cerebral cortex (10).

Several lines of evidence showed antioxidant and neuroprotective effects of TQ.

Administration of TQ significantly decreased brain damage following cerebral ischemia-reperfusion injury through reduction of lipid peroxidation (19).

In another study, TQ elevated GSH content, SOD and CAT activity in hippocampus and reduced injury induced by transient forebrain ischemia (20). Chronic toluene exposure induced neuron apoptosis in frontal cortex while TQ treatment caused morphologic improvement (23).

According to our results, administration of TQ in a dose-dependent manner dramatically diminished level of MDA in cerebral cortex in comparison to ACR treated animals.

For evaluation the effect of time on TQ protective effects, TQ was used simultaneously with ACR or used 1 week before starting of ACR administration and continued during treatment with ACR. Obtained results clearly showed that there is no significant difference between two protocols.

In the current study, vitamin E was chosen as a positive control because it seems that neuroprotective effects of vitamin E may be due to its antioxidant activity (26, 27). The results indicated that there is no difference between TQ (10 mg/kg) and vitamin E in inhibition of lipid peroxidation in cerebral cortex and improving the severe gait abnormalities in animals.

Because of the critical role of oxidative stress in ACR-induced neurotoxicity, it suggests that the protective effects of TQ in this model may be at least because of its antioxidant activity.

Conclusion

TQ exhibited protective effects against ACR-induced neurotoxicity in Wistar rats through reduction of lipid peroxidation in cerebral cortex.

Initiation of supplementation 1 week before or simultaneous with ACR administration had same effects in this model. To our knowledge, this is the first report on protective effect of TQ in ACR-induced neurotoxicity in Wistar rats.

Acknowledgment

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