

## The Effects of Thymoquinone on Nitric Oxide and Superoxide Dismutase Levels in a Rat Model of Diazinon-induced Brain Damage

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**ABSTRACT** Diazinon (DI) is an organophosphate. Thymoquinone (TQ) is produced from *Nigella sativa*. This study was a biochemical investigation of the protective properties of TQ as a supportive treatment in brain damage caused by diazinon. Rats were divided into four groups of eight, as follows: Group 1, control; group 2, DI; group 3, DI + TQ; and group 4, TQ. Groups 2 and 3 received DI. Groups 3 and 4 received TQ. In group 2, NO levels were higher and SOD levels were lower compared to the control group. In groups 3 and 4, NO levels were lower and SOD levels were higher than in either the control or group 2. In group 4, DNA damage was lower than in group 2, but the difference between groups 2 and 3 was not significant. The results suggest that TQ, because of its antioxidant properties, can be used to treat oxidative stress caused by DI intoxication.

### INTRODUCTION

Diazinon (DI) is a non-systemic organophosphate insecticide (IUPAC name: O, O-Diethyl O-[4-methyl-6-(propan-2-yl)pyrimidin-2-yl] phosphorothioate, INN - Dimpylate) (Colovic et al. 2015; Beydilli et al. 2015). Due to its highly acute toxicity to their target organisms and rapid biodegradation, organophosphate insecticides (OPIs) are widely used throughout the world, including in Turkey (Yilmaz et al. 2012). OPIs are readily absorbed by cellular membranes, in which their main mechanism of action is the inhibition of cholinesterases, including acetyl cholinesterase. The OPI diazinon (DI) is inexpensive and

thus commonly available but proper control of its use is often lacking (Colovic et al. 2015; Beydilli et al. 2015; Yilmaz et al. 2012).

In humans, the skin and the pulmonary and gastrointestinal systems rapidly absorb DI, such that acutely it is highly toxic, causing damage to the central nervous system and liver and often leading to death (Colovic et al. 2015; Beydilli et al. 2015; Lari et al. 2015; Elsaid et al. 2015; El-Demerdash et al. 2014). In rural areas, there are frequent cases in which DI has been used to commit suicide. Experimental studies on the cellular mechanism of DI toxicity, both acute and chronic, have shown that it causes oxidative stress and DNA damage (Jafari et al. 2012; Yassa et al. 2011; Beydilli et al. 2015).

*Nigella sativa*, popularly known as fennel flower, grows as an herbaceous plant along the Mediterranean coast and in Asia. It has long been used in traditional medicine, as a remedy for colds, headaches, rheumatism, and other ailments. An analysis of its volatile oils showed

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that the main active ingredient is thymoquinone (TQ). The anti-diabetic, antioxidant, antihistamine, anti-inflammatory, anti-tumoural and immunomodulatory properties of TQ and its effects on blood clotting have been demonstrated in several studies (Güllü and Avci 2013; Bulca 2015; Darakhshan et al. 2015). However, whether TQ has protective effects on DI-induced brain damage has not been determined. Thus, in this study, a rat model of DI-induced brain damage was used to analyze the protective effects of TQ and its mechanism of action in DI toxicity. The results suggest that in patients admitted to the emergency department with DI poisoning, TQ represents an effective supportive treatment to inhibit oxidative injury.

## MATERIAL AND METHODS

The study was conducted in the veterinary laboratory of Süleyman Demirel University (Isparta, Turkey). Wistar Albino female rats (aged 12 weeks, 200±20 g) were housed in standard laboratory conditions (25±2°C and 40–70% relative humidity) with a 12-hour day/night lighting cycle (7 am to 7 pm) throughout the experimental period and allowed a commercial standard rat diet (Abalioglu Yem Sanayi, Denizli, Turkey) and water ad libitum. All rats were allowed to acclimate for 1 week prior to the initiation of any experiment. The Ethical Council of Süleyman Demirel University of Medical Sciences (Isparta, Turkey) approved all protocols used with these rats in the studies herein. The experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals (DHEW Publication (NIH) 8523, 1985).

Thirty-two 12-week-old Wistar Albino female rats (200±20 g) were divided into four groups of eight as follows: Group 1, non-treated control; group 2, DI; group 3, DI + TQ; and group 4, TQ. Rats in groups 2 and 3 were given a single oral dose of 335 mg DI/kg. After one week, rats in groups 3 and 4 were orally administered 10 mg TQ/kg per day for 7 days. At the end of day 7, all rats were killed by decapitation under anesthesia.

At the end of the experiment, all rats were anesthetized under an intraperitoneal injection of ketamine/xylazine (60 mg/kg and 6 mg/kg, respectively). Blood samples were taken from intracardiac on the sterile tubes. Blood samples were centrifuged and serum was separated. The

blood was centrifuged at 2000 × G for 15 minutes, at 4°C. The top yellow serum layer was pipetted off, without disturbing the white buffy layer.

The rats were quickly decapitated. Brain tissue samples were taken for biochemical analysis. Rat brains were removed immediately and washed with phosphate buffer solution (PBS) (pH = 7.4). They were placed in aluminum foil and immediately kept in the refrigerator at 80°C.

Blood and tissue samples were collected and stored at -80°C until used in the experiments. Levels of the oxidant nitric oxide (NO) and the antioxidant superoxide dismutase (SOD) in homogenized brain tissue were determined by ELISA.

## Determination of Nitric Oxide Level

The tissue was homogenized in PBS (pH 7.4) and centrifuged at 10000 × G for 20 minutes to create the supernatant. Total NO assay was performed by spectrophotometry at 540 nm using the nitrate/nitrite colorimetric assay kit (Cayman, Ann Arbor, Michigan USA) in Bio-Tek ELx-800. The assay was based on nitrate and nitrite determinations. The nitrate and nitrite are the stable end products of the reaction of NO with molecular oxygen. The total accumulation of nitrate and nitrite in brain tissue was measured. The results were expressed as micromoles per gram (µm/g) protein for brain tissue.

## Determination of Superoxide Dismutase Activity

The tissue was homogenized in 5-10 mL cold buffer (50 mM Tris-HCl, pH 7.5, 5 mM EDTA and 1 mM DTT (Dithiothreitol) per tissue. Then, it was centrifuged at 10,000 × G for 15 minutes, at 4°C. The supernatant was removed after centrifugation. The SOD was determined via Cayman's Superoxide Dismutase assay kit (Cayman Chemical Co., Ann Arbor, MI, USA) in Bio-Tek ELx-800 (Winooski, USA). The detection of superoxide radicals was generated by xanthine oxidase and hypoxanthine. One unit of SOD is defined as the amount of enzyme required to exhibit fifty percent dismutation of the superoxide radical. The dynamic range of the kits is 0.005 - 0.05 U/mL SOD. Recommended by the company for measuring formulation, the SOD was calculated by applying SOD values. Enzyme activities re-

sults were reported as units per gram protein (U/mg) in the brain tissue.

DNA breaks were measured in the serum by the comet assay. In the comet assay, the rate of DNA damage was determined according to the Duncan method, with the minimum difference between two groups calculated using a Duncan table. Group averages were ranked and then evaluated on the basis of the gaps between the respective values.

Statistical analyses were conducted using the SPSS, version 15.0. The Kruskal-Wallis test was used to determine significant differences within each group; differences between groups were analyzed using the Mann Whitney U-test. Significance was defined as  $p < 0.05$ .

## RESULTS

Nitric oxide levels in the homogenized brain tissue of DI-treated rats in group 2 were significantly higher than those in the untreated control group ( $p < 0.01$ ). In the brain tissues of group 3 rats (DI + TQ), NO levels were significantly lower than in those of group 2 ( $p < 0.01$ ). NO levels were also significantly lower in group 4 (TQ) than in the control group 1 ( $p < 0.01$ ) (Table 1).

Similar comparisons showed that SOD levels were lower in group 2 (DI) than in the control group 1 ( $p < 0.01$ ) but significantly higher in group 3 (DI+TQ) than in group 2 ( $p < 0.01$ ) and in group 4 (TQ) than in group 1 ( $p < 0.01$ ) (Table 1).

The rate of DNA damage in serum was determined using the Duncan method. In group 1 (control), the average DNA damage score was  $15.00 \pm 3.00$ .

In group 2 (DI), the average DNA damage score was  $26.00 \pm 5.29$ , significantly higher than that of group 1 ( $p < 0.05$ ). However, there was no significant difference in DNA damage scores between group 3 (DI+TQ group) and group 2, whereas in group 4 (TQ) there was a significant decrease in DNA damage compared to that in group 2 ( $p < 0.05$ ) (Table 2).

**Table 1: NO and SOD levels in rat brain tissue**

	Group 1 (Control)	Group 2 (Diazinon)	Group 3 (Diazinon + Thymoquinone)	Group 4 (Thymoquinone)
NO ( $\mu\text{m/g}$ )	$0.669 \pm 0.318$	$1.852 \pm 1.036^a$	$0.287 \pm 0.94^{a,b}$	$0.245 \pm 0.32^a$
SOD (U/mg)	$0.314 \pm 0.04$	$0.126 \pm 0.07^a$	$0.977 \pm 0.23^{a,b}$	$0.591 \pm 0.21^a$

*a*:  $p < 0.01$  compared to the control group

*b*:  $p < 0.01$  compared to the diazinon group

**Table 2: Results of the comet assay of DNA damage.**

Group	DNA damage (Arbitrary units $\pm$ SD)
1 (control)	$15.00 \pm 3.00$
2 (DI)	$26.00 \pm 5.29^a$
3 (DI+TQ))	$24.66 \pm 1.15$
4 (TQ)	$18.66 \pm 3.05^b$

*a*:  $p < 0.01$  compared to the control group

*b*:  $p < 0.01$  compared to the diazinon group

## DISCUSSION

Organophosphate pesticides such as the broad-spectrum insecticide DI are used extensively in many agricultural areas throughout the world, as well as in home gardens. However, when OPIs are applied inappropriately the residues can remain on vegetables and other plants, with potentially harmful effects on the consumers of these products (15). Moreover, especially in rural areas, OPIs have been consumed intentionally to commit suicide (Beydilli et al. 2015; Yilmaz et al. 2012).

The toxic effects of OPIs on living organisms are caused by the induction of free-radical formation and the inhibition of cellular cholinesterases. DI is an inhibitor of acetylcholinesterase, an enzyme present at nerve junctions and important in neuronal signaling (Ogut et al. 2015; Singh et al. 2015).

DI is delivered to the body through the gastrointestinal tract, skin contact, or inhalation and excreted through the urinary system. Its genotoxic, cytotoxic, hepatotoxic, embryotoxic, neurotoxic, and adrenotoxic effects have been demonstrated. OPI-induced damage is mediated by the increased production of oxygen free radicals (Colovic et al. 2015; Beydilli et al. 2015; Lari et al. 2015; Elsaid et al. 2015; El-Demerdash et al. 2014), with deleterious effects on the central nervous system, the cardiovascular, urogenital, metabolic, and endocrine systems, and neuro-

muscular junctions of both humans and animals (Yildirim and Özdamar 2012). Oxidative-stress-induced brain damage has been demonstrated (Colovic et al. 2015; Lari et al. 2015; Elsaid et al. 2015; El-Demerdash et al. 2014; Yassa et al. 2011; Jafari et al. 2012). These findings are consistent with those of the present study, in which the levels of NO, an indicator of lipid peroxidation, were significantly higher in the DI group than in the untreated control group.

Free radicals are electrically charged compounds that pass through the cell membrane to react with cellular nucleic acids, proteins, including enzymes, and other cellular constituents (Harzallah et al. 2012; Salem 2005). They include reactive oxygen species (ROS) such as superoxide and hydroxyl radicals, sulphur-centred thiol radicals, and carbon-centered radicals, such as trichloromethyl and NO. The antioxidant properties of TQ have been attributed to its ability to scavenge ROS and inhibit the synthesis of 5-hydroxyeicosatetraenoic acid and 5-lipoxygenase, both of which may play a role in disease (Sheikh et al. 2012; Radad et al. 2014; Vafaei et al. 2015). TQ mediates its inhibitory effect on NO production via reduction of iNOS mRNA and protein expressions (El-Mahmoudy et al. 2002). In doxorubicin-induced nephropathy, TQ was shown to have antioxidant properties, by preventing lipid peroxidation, which in turn suppressed nephropathy development (Elsherbiny and El-Sherbiny 2014; Badary et al. 2000). TQ is also more active than synthetic tert butyl hydroquinone as a superoxide anion scavenger.

The results suggest that the protective effects of TQ in DI-induced brain damage caused by oxidative stress reflect its antioxidant activity (Colovic et al. 2015; Yilmaz et al. 2012). Specifically, NO levels were lower and SOD levels were higher in the brains of DI+TQ animals than in rats treated with DI alone. These findings are in agreement with those of other studies of the neuroprotective and antioxidant effects of TQ (Sheikh et al. 2012; Radad et al. 2014; Vafaei et al. 2015; Salem et al. 2005). In addition, in a previous study by Yilmaz et al. (2012), the toxic effects of DI in rats were diminished by the administration of vitamin E and vitamin C, the antioxidant effects of which are well established.

As shown by the comet assay, rats given a single oral dose of DI had a significantly higher average DNA damage score than the untreated control group. However, there was no significant

difference in the amount of DNA damage between the DI and DI+TQ groups. Thus, at least at the dose tested, TQ was unable to inhibit DNA damage, although in group 4, that is, rats not previously administered DI but given TQ alone, DNA damage was significantly lower than in the DI group ( $p < 0.05$ ). When this data is analyzed, it was found that DNA damage occurs before Timokinon protects DNA from free radicals but the researchers consider not reverse the DNA damage caused by diazinon. In the study by Harzallah et al., Timokinon 80 mg / kg / day statistically significantly higher doses have been found to prevent DNA damage (Harzallah et al. 2012). This study involved giving the rats a low dose (10 mg / kg / day). Therefore, Timokinon could not prevent DNA damage. These results suggest that while TQ can prevent DNA damage by free radicals, it is insufficient to protect against DI-induced DNA breaks.

## CONCLUSION

Thymoquinone may be useful in the treatment of diazinon induced poisoning, based on the antioxidant properties of this volatile oil, and perhaps also in the mitigation of drug-related side effects. To better understand the effects of Thymoquinone on metabolic pathways, additional research, including different doses and exposure times, is needed.

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