

Evaluation of attenuative effect of tert-butylhydroquinone against diazinon-induced oxidative stress on hematological indices in male Wistar rats

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Abstract. Diazinon is an organophosphate toxicant that has been identified to induce oxidative stress within biological systems through altering biochemical and hematological indices, by generating free radicals while deteriorating the cellular antioxidant defense system. The present study was designed to evaluate the effectiveness of tert-butylhydroquinone (TBHQ), a synthetic food additive, in serving as an antioxidant against sub-lethal diazinon-induced oxidative stress in male Wistar rats. Animals were randomly divided into 4 groups: Control (treated with corn oil as a vehicle), diazinon (10 mg/kg/day), TBHQ (0.028 g/kg of diet) and a combination group of TBHQ (0.028 g/kg of diet) plus diazinon (10 mg/kg/day). All animals were treated orally once a day by gastric tube and treatments were continued for 7 weeks. Hematological indices, including red blood cell (RBC) indices and white blood cell (WBC) and platelet counts, were measured at the end of the treatment schedule. On comparison of the treatment groups with the untreated control group, RBC count, hemoglobin (Hb), hematocrit (Hct), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were significantly decreased in the diazinon and TBHQ+diazinon groups, while MCHC in the TBHQ group and MCV in the diazinon and TBHQ+diazinon groups were significantly increased. When the diazinon and TBHQ+diazinon groups were compared with the TBHQ group, RBC, Hb, Hct, and MCHC were significantly decreased whereas MCV was increased in both groups. Additionally, when comparing the TBHQ+diazinon group with the diazinon group, MCHC was determined to be significantly decreased. The results of the current experiments suggested

that TBHQ could not efficiently protect blood cells against diazinon toxicity.

Introduction

Oxidative stress is generally described as any biochemical or physiological condition that results in an imbalance between the rate of reactive oxygen species (ROS) production and the ability of body antioxidant systems to overcome the disruption caused by these chemically reactive radicals (1). The most common ROS are hypochlorite ion, superoxide anion, hydrogen peroxide and in particular hydroxyl radical, which is considered the most reactive (2). Free radicals typically interact with polyunsaturated fatty acid residues of cell membrane phospholipids, forming lipid peroxides that can further produce mutagenic and carcinogenic malondialdehyde (MDA) in addition to cyclic DNA adducts (3,4). Additionally, oxidative stress has potent effects in the pathogenesis of several clinical conditions including hypertension (5), coronary heart diseases (6) and both type 1 and type 2 diabetes mellitus (7). Regarding the nature of ROS, some are notably toxic and are rapidly detoxicated to less harmful metabolites by a range of cell mechanisms that are either enzymatic or non-enzymatic (8). This conversion is key in affected cells to enable normalization of cell function and metabolism (9).

Organophosphate (OP) compounds consist of a variety of chemicals that are the most toxic among all pesticides to vertebrates and humans (10). However, OPs are still widely used in certain third world countries for agricultural or veterinary interventions as solvents or extreme pressure additives (11,12). In the last twenty years the improper use of OPs has led to toxic resistance, which is of major concern to the environment, animals and human populations (13). Additionally, it has been reported that residual quantities of OPs may be detected in plants, tissues of organisms and foods products (7). OPs are generally rapidly absorbed by the skin or mucous membranes of the oral cavity during inhalation (8), which mediates the onset of adverse reactions. Furthermore, OPs are considered to have damaging effects on the urinary (14) and reproductive (15) systems as for other vital organs.

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The neurobehavioral development of fetuses and children is considered to be one of the disorders with an undetermined association with OP exposure even at very low levels. OPs are classified into four principal groups, namely as herbicides (trichlorfon), ophthalmic glaucoma agents, certain types of nerve gases (VX and sarin) and most notably, insecticides, for which there is a marked number (including diazinon, malathion and parathion) (16). Although it has been demonstrated that OPs inhibit the activity of acetylcholinesterase (AChE), the main enzyme responsible for acetylcholine (ACh) degradation (17), further study revealed that the toxic consequences of OP exposure may be due to induction of oxidative stress (18). The acute toxicity of OPs manifests as cholinergic damage, which is associated with irreversible covalent inhibition of cholinesterase activity in the central nervous system (19). As a result, accumulated ACh is not degraded and nicotinic receptors are activated, and consequently cell death is prompted (20).

Diazinon [O,O-diethyl-O-(2-isopropyl-6-methyl-4-pyrimidinyl)phosphorothioate] is a colorless liquid and also a thiophosphoric acid derivative that is ordinarily used to control pests and insects (21). There is a range of data indicating the association between diazinon toxicity and altered liver enzyme function and biochemical and hematological parameters (22-24). As mentioned, the main mechanism by which diazinon induces acute intoxication is AChE inhibition, which causes an abnormal accumulation of ACh in the synaptic cleft (25). Exposure to sub-lethal doses of diazinon is associated with chronic toxicity, and ultimately elevated lipid peroxidation and decreased antioxidant enzyme activity (10,26). Furthermore, since diazinon is fat soluble, prolonged toxicity should be considered if a substantial quantity of diazinon is stored in fatty tissues (27). By contrast, it has been established that antioxidants as scavenger substances are capable of neutralizing ROS or inhibiting free radical formation, and thus effectively protect cells against oxidative stress-inducing agents including OPs (28). There are two major types of antioxidants: Enzymatic and non-enzymatic. Over previous years, vitamins C and E and numerous other natural and synthetic compounds functioning as non-enzymatic antioxidants have been used against cellular oxidative stress induction caused by OP substances (29).

There are a number of synthetic antioxidants that are permitted by the US Food and Drug Administration for use in food products (30). Among edible synthetic antioxidants, tert-butylhydroquinone (TBHQ) is primarily used as a food additive for unsaturated vegetable oils (31). TBHQ is considered to mainly function via induction of nuclear translocation of the transcription factor NF-E2-related factor 2 (Nrf2), which in turn potentially regulates the gene expression of certain antioxidant enzymes, including heme oxygenase-1 and glutathione S-transferase (32).

Despite the importance of OP toxicity as a health issue and the risk of constant exposure of farmers to OP substances, particularly diazinon, there is no sufficient data regarding the combined effects of diazinon and TBHQ on hematological indices alteration. Therefore, the present study aimed to evaluate the effects of sub-lethal doses of the OP insecticide diazinon on hematological parameters, and to investigate the possible attenuative role of TBHQ against ROS production in Wistar rats.

Materials and methods

Chemicals. All chemicals used were of pure analytical grade. Diazinon, purity 99%, was obtained from Merck KGaA (Darmstadt, Germany) and TBHQ was supplied by Sigma-Aldrich (Merck KGaA).

Animals. A total of 28 adult male Wistar rats weighing 250-300 g (mean age, 18 ± 2 weeks old), purchased from the central animal house facility at Mashhad University of Medical Sciences (Mashhad, Iran), were utilized for the current cross-sectional experimental study. The rats were caged (with a mean number of 4 animals per cage; maximum, $n=6$) with paddy husk bedding under a 12-h light/dark cycle (light period from 6.30 a.m. to 6.30 p.m.), $23 \pm 2^\circ\text{C}$ and 60% humidity. The rats were acclimatized for at least 7 days prior to randomizing into experimental groups. The animals were permitted access to a commercial pellet diet (manufactured in Javaneh Khorasan, Mashhad, Iran) and tap water *ad libitum*. All animals received human care and the protocols of the experiment were approved by the Animal Welfare Committee of the School of Veterinary Medicine, Ferdowsi University of Mashhad (Mashhad, Iran).

Experimental protocol and drugs. The 28 male Wistar rats were categorized randomly in 4 equal-sized groups ($n=7$ per group) comprising of a control group and three experimental groups, as follows: Group 1 (Control), which received corn oil as a vehicle for both TBHQ and diazinon, once a day via gavage tube; group 2 (diazinon-treated group), which was administered diazinon (10 mg/kg/day) in corn oil, once a day via gastric tube, with this selected dose having been used in previous studies and demonstrated efficacy in altering the biochemical and hematological parameters of male Wistar rats (24,29); group 3 (TBHQ-treated group), in which TBHQ was orally administered at a dose of 0.028 g/kg of diet (in corn oil), which has previously been indicated to have antioxidant effects (33); and group 4 (TBHQ+diazinon), in which diazinon and TBHQ (also in corn oil) were orally administered at the aforementioned doses (10 mg/kg/day of diazinon and 0.028 g/kg of diet of TBHQ) once a day via gavage tube.

In attempt to approximate the average daily diet consumption in grams, the food intake of rats was assessed over the 7 days of acclimatization by calculating the difference between intact pre-weighed food and the quantity of food remaining after 24 h in cages. All rats were non-fasted by the time of oral administration (between 10 and 11 a.m. in the morning). The first treatment day was appointed the first day of the experiment. The volume of treatment solution was equal for all the groups.

At the end of day 49, animals were euthanized. Whole blood samples were immediately collected through cardiac puncture (up to 7 ml was drawn from each rat). Using a Bayer-Advia 120 hematology analyzer (Bayer Corporation, Whippany, NJ, USA), the blood samples with anti-coagulant EDTA were instantly analyzed to evaluate the main hematological indices, namely red blood cell (RBC) count, hemoglobin (Hb) concentration, hematocrit (Hct) levels, white blood cell (WBC) count, mean corpuscular Hb (MCH), mean corpuscular volume (MCV), mean corpuscular Hb concentration (MCHC) and platelet count.

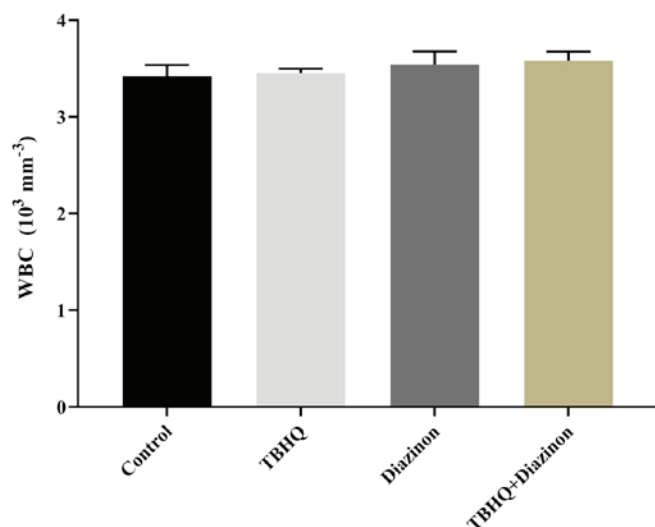


Figure 1. Mean values of WBC count in control, TBHQ and/or diazinon-treated rats. WBC, white blood cell; TBHQ, tert-butylhydroquinone.

Statistical analysis. Statistical analysis was conducted using SPSS version 22 (IBM Corp., Armonk, NY, USA). One-way analysis of variance with post hoc Tukey's tests were used to determine the significance of differences, with $P < 0.05$ considered to indicate statistical significance. The F-test was performed to determine the normal distribution of variances between groups. The results are presented as the mean \pm standard deviation obtained from the 7 animals in each group.

Results

Comparison of hematological parameters. The results of hematological parameters in the current study were assessed according to three comparisons: i) Compared with the untreated control group; ii) compared with the TBHQ-treated group; and iii) compared with the diazinon-treated group.

WBC count. Comparison of WBC count between the four groups in the current study identified no statistically significant differences, although TBHQ led to a marginal increase in WBC count compared with the untreated control group. This enhancement was observed in the diazinon and combination groups with increased intensity (Fig. 1).

RBC, Hb and Hct. Notable changes were observed in RBC count, Hb content and Hct levels in erythrocytes. The number of RBCs was significantly decreased in the diazinon and TBHQ+ diazinon groups compared with in the untreated control and TBHQ groups ($P < 0.001$). Hb and Hct levels were also significantly decreased in the diazinon and TBHQ+ diazinon groups compared with in the control and TBHQ groups ($P < 0.001$; Fig. 2).

MCV, MCH and MCHC. Comparing the untreated control group with the other groups, significant alterations were identified in MCV [diazinon group vs. control group ($P < 0.001$) and TBHQ+ diazinon group vs. control group ($P < 0.001$)], MCH [diazinon group vs. control group ($P < 0.05$) and TBHQ+ diazinon group vs. control group ($P < 0.05$)] and MCHC [TBHQ group vs. control group ($P < 0.05$), diazinon

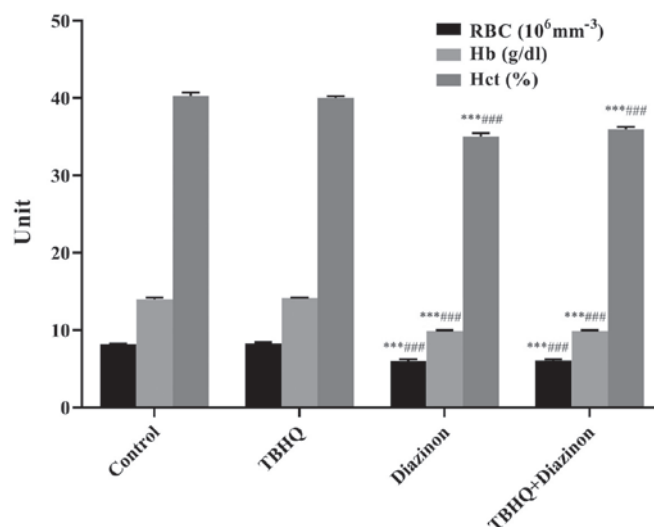


Figure 2. Mean values of RBC, Hb and Hct in control, TBHQ and/or diazinon-treated rats. *** $P < 0.001$ vs. control; ### $P < 0.001$ vs. TBHQ. RBC, red blood cell; Hb, hemoglobin; Hct, hematocrit; TBHQ, tert-butylhydroquinone.

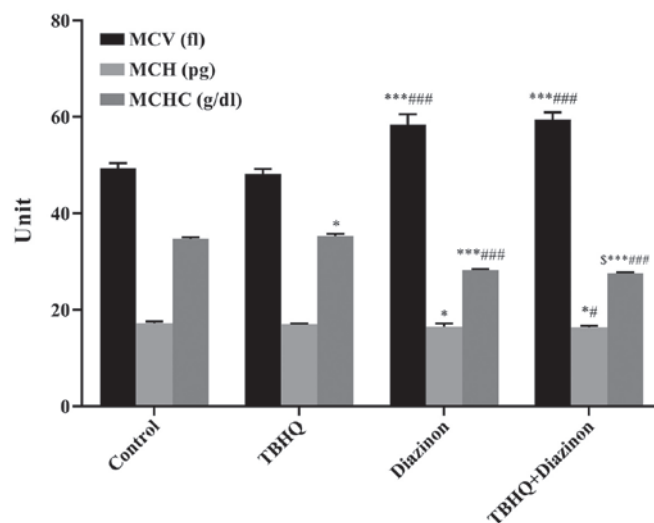


Figure 3. Mean values of MCV, MCH and MCHC in control, TBHQ and/or diazinon-treated rats. * $P < 0.05$, *** $P < 0.001$ vs. control; # $P < 0.05$, ### $P < 0.001$ vs. TBHQ; § $P < 0.05$ vs. Diazinon. MCV, mean cell volume; MCH, mean cell hemoglobin; MCHC, mean cell hemoglobin concentration; TBHQ, tert-butylhydroquinone.

group vs. control group ($P < 0.001$) and TBHQ+ diazinon group vs. control group ($P < 0.001$)). Significant differences were also identified between the diazinon groups (single and combined treatments) and TBHQ group concerning MCV [diazinon group vs. TBHQ group ($P < 0.001$) and TBHQ+ diazinon group vs. TBHQ group ($P < 0.001$)], MCH [TBHQ+ diazinon group vs. TBHQ group ($P < 0.05$)] and MCHC [diazinon group vs. TBHQ group ($P < 0.001$) and TBHQ+ diazinon group vs. TBHQ group ($P < 0.001$)]. MCHC was the only parameter that significantly differed between the TBHQ+ diazinon and diazinon groups ($P < 0.05$), being significantly decreased in the TBHQ+ diazinon group (Fig. 3).

Platelet count. There were no significant differences in platelet number between all four groups ($P > 0.05$; Fig. 4).

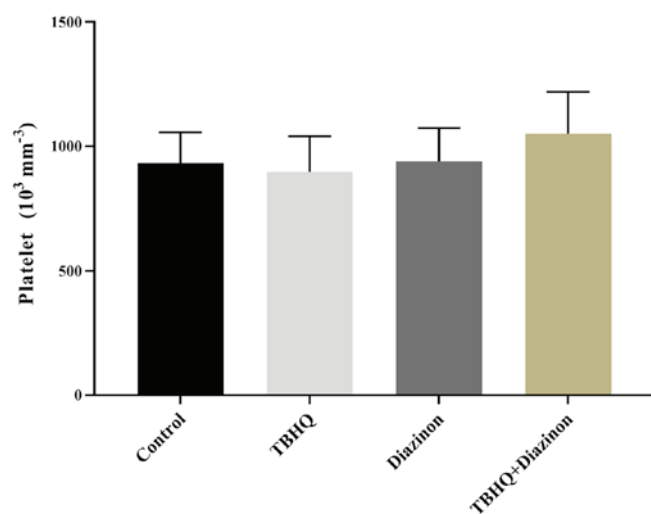


Figure 4. Mean values of platelet count in control, TBHQ and/or diazinon-treated rats. TBHQ, tert-butylhydroquinone.

Discussion

OP toxicity is considered to be among the most important challenges currently faced by agricultural environments, and biological environments in general. Over previous years, a number of types of pesticides have been used for pest and insect control, and the use of OPs has been reported to have among the most potent effects (34). In this regard, OPs are essential for sufficient agricultural yield, and thus their application still continues in certain developing countries (35). Diazinon is widely used as an insecticide for plant protection, and also serves as an ectoparasitic agent for parasitic control in sheep (36). As a consequence, broad distribution of OPs including diazinon in the environment correlates with onset of toxic effects in land and marine animals (37,38). Alongside the natural antioxidants, permitted concentrations of synthetic antioxidants have been demonstrated to exert protective effects against the oxidative stress-induced cytotoxicity (39). In the present study, the attenuative effect of TBHQ against diazinon-induced toxicity with regard to hematological indices was evaluated in 28 male Wistar rats, in which mild oxidative damage was observed following exposure to sub-lethal levels of diazinon, indicated by reductions in RBC count, Hb concentration and Hct level.

Primary hematological indices include WBC, RBC and platelet counts. Other parameters including Hb, Hct, MCV, MCH and MCHC provide specific information on RBC characteristics such as size and morphology in vertebrates (40). In the current study, TBHQ, Diazinon, or their combination had no significant effect on WBC and platelet numbers, although the compounds had marked influence on RBC-related indices. Notably, RBC count was decreased in male Wistar rats following exposure to diazinon alone or in combination with TBHQ, compared with in the control and TBHQ groups. Reductions in Hb and Hct were also observed. Other parameters of RBCs including MCV, MCH and MCHC were also influenced following exposure of the rats to the compounds: MCV was increased following treatment with Diazinon and TBHQ+Diazinon compared with in the untreated and TBHQ

groups; MCH was marginally decreased in the diazinon group compared with in the untreated group, and in the TBHQ+Diazinon groups compared with in the untreated and TBHQ groups; and MCHC was significantly lower in the untreated group vs. TBHQ group and TBHQ+Diazinon group vs. diazinon group. Additionally, MCHC was reduced in the diazinon and TBHQ+Diazinon groups compared with in the untreated and TBHQ groups. A possible mechanism for the reduction in RBC count is that chronic intoxication induced by diazinon leads to oxidation of the membrane phospholipids of the RBC cells, subsequently decreasing Hb and Hct and effecting related indices including MCV, MCH, and MCHC; while the antioxidant of the present study (TBHQ) did not efficiently protect the morphology of RBCs against 10 mg/kg/day diazinon. Regarding WBC and PLT counts, the related mechanism cannot be concluded due to contradictory results of previous studies mentioned below.

Many investigations have been conducted concerning the effect of diazinon toxicity on hematological indices, which have yielded contradictory data (41,42); nevertheless, the current results do not contradict the findings of most previous investigations mentioned above. However, to the best of our knowledge, there is no published report on utilization of TBHQ as an antioxidant substance and its combination with diazinon in effecting hematological parameters.

According to previous experiments performed by Kalender *et al* (22), all RBC parameters tested except MCH were altered in rats exposed to diazinon compared with in an untreated group at 1, 4 and 7 weeks after treatment. A study on the effects of Basudin 600 EW (an active substance of diazinon) in common carp fish (*Cyprinus carpio* L.) demonstrated that this compound did not exert significant effect on MCV, MCH or MCHC, but significantly decreased leukocyte and lymphocyte count while increasing the absolute count of developmental forms of neutrophil granulocytes including myelocytes and metamyelocytes (41). Another similar study conducted by Ahmad (43) highlighted the effect of diazinon exposure toxicity in common carp, manifested as increases in RBC, Hb and Hct. Effects of diazinon on the hematological parameters of fry rainbow trout (*Oncorhynchus mykiss*) have also been revealed, in that sub-lethal doses of the OP substance led to a significant decrease in RBC and Hb, and increased MCV and MCH in a diazinon-exposed group in comparison with untreated groups (42). The results of a study by Yassa *et al* (44) indicated a significant decrease in Hb concentration and RBC number in a diazinon group from the third week of the experiment compared with levels in untreated rats. Banaee *et al* (45) identified that diazinon treatment caused an increase in RBC, Hb and Hct parameters while decreasing MCV and MCH compared with in an untreated group in common carp. Various doses of diazinon led to reduction in RBC count, Hb concentration and other indices including Hct, MCV, MCH and MCHC in fingerling European catfish (*Silurus glanis* L.) in an investigation performed by Köprüci *et al* (46). Additionally, other studies have also demonstrated alterations in hematological indices in different species including rat (47), African catfish (*Clarias gariepinus*) (48) and Indian carp [*Cirrhinus mrigala* (Hamilton)] (49).

Regarding improvements to the current study, extending the number of rats in each experimental group, using higher

doses of TBHQ, and assessment of oxidative stress-related markers by methods including monitoring of their gene expression patterns may be useful in further investigations to acquire more comprehensive and conclusive results.

Nevertheless, the present data suggest that TBHQ as a novel synthetic antioxidant could not efficaciously attenuate the effects of sub-lethal exposure of diazinon on hematological indices in male Wistar rats, despite attenuating certain red cell indices affected by diazinon. The use of higher doses of TBHQ may be necessary to acquire a more convincing outcome.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

SS designed the experiment protocol and established the structure and content of the manuscript. HG analyzed the data. SS and SZ wrote the first draft of the manuscript. HG and SS contributed to the writing of the manuscript and verified the manuscript results and conclusions. HG and SZ made critical revisions and approved the final version. All authors reviewed and approved the final manuscript.

Ethics approval and consent to participate

All animal protocols were approved by the Animal Welfare Committee of the School of Veterinary Medicine, Ferdowsi University of Mashhad (Mashhad, Iran).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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