Tert-butylhydroquinone (TBHQ) improves antioxidant status in rat tissues following chronic diazinon intoxication

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Received: October 8, 2014                 Accepted: January 17, 2015

Abstract

Considering that the involvement of oxidative stress has been implicated in the toxicity of organophosphate insecticides, the objective of the present study was to investigate antioxidants and oxidative stress markers in the liver and kidney of Wistar rats treated with chronic doses of diazinon. In addition, the effect of Tert-butylhydroquinone (TBHQ), a food-additive antioxidant, on attenuation of diazinon-induced oxidative stress was evaluated. 28 rats were randomly assigned to 1 of 4 treatment groups: diazinon (10 mg/kg BW, once a day; n=7), TBHQ (0.028 g/kg of diet, once a day; n=7), TBHQ + diazinon (diazinon; 10 mg/kg BW, once a day + TBHQ; 0.028 g/kg of diet, once a day; n=7) and control (corn oil, as vehicle of diazinon and TBHQ, n=7). TBHQ, diazinon and corn oil were given to rats orally via gavage for 7 weeks. Total thiol groups, ferric reducing antioxidant power (FRAP) and malondialdehyde (MDA) levels in liver and kidney tissues were investigated at the end of 7th week. Treatment with diazinon significantly increased MDA level, as a marker of lipid peroxidation, in the kidney (p<0.05). Co-administration of TBHQ with diazinon significantly increased total thiol groups and FRAP levels in liver and kidney (p<0.05). However, TBHQ did not reduce lipid peroxidation in the tissues. The results of the present study showed that administration of TBHQ at a dose of 0.028 g/kg of diet, increased antioxidant capacity of tissues of rats treated with diazinon, though this increase was not sufficiently effective to reduce lipid peroxidation caused by diazinon. Higher amounts of TBHQ might be more effective in attenuation of diazinon-induced lipid peroxidation.

Keywords: diazinon, oxidative stress, TBHQ, rat

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Introduction

Organophosphate compounds (OPs) are commonly used as insecticides, and are generally the most toxic of all pesticides to vertebrate animals (Dawood Shah and Iqbal, 2010). It is well established that OPs cause inhibition of acetylcholinesterase activity in the target tissues (Buyukokuroglu et al., 2008). However, the toxic effect of OPs is not limited to inhibition of cholinesterase. It has recently been reported that oxidative stress may be an important factor in OP-induced toxicity (Lu et al., 2012). The imbalance between generation of reactive oxygen species (ROS) and antioxidant mechanisms in the body is called oxidative stress, which has important health implications (Heidarpour et al., 2012). ROS are produced as the result of the metabolism of OPs by cytochrome P450s. The disturbance in the cell redox system is another mechanism implicated in the generation of ROS in OPs exposure. In addition, the exposure to OPs induces hyperglycemia leading to increase of non-enzymatic glycation by the binding of glucose or by-products to proteins and formation of advanced glycation end products. Glycated proteins activate specific membrane receptors and induce an intracellular oxidative stress (Lukaszewicz-Hussain, 2010). ROS can be detoxified by enzymatic and non-enzymatic antioxidants, which are essential for the conversion of ROS to harmless metabolites as well as to protect and restore normal cellular metabolism and functions (El-Shenawy et al., 2010). OPs pesticides have been reported to decrease the level of antioxidants (Muniz et al., 2008). Lipid peroxidation is a complex process resulting from ROS reactions in biological membranes, which are rich in polyunsaturated fatty acids (Oruc and Usta, 2007). One of the molecular mechanisms of the toxicity of some pesticides seems to be lipid peroxidation (Mansour and Mossa, 2009).

Diazinon (o,o-diethyl-o-[2-isopropyl-6-methyl-4-pyrimidinyl]phosphorothioate) is one of the most commonly used OPs in the world (Kalender et al., 2005). Diazinon causes changes in liver enzymes and biochemical indices and affects mitochondrial membrane transportation and cytochrome P450 system in hepatocytes (Nakagawa and Moore, 1999; Sams et al., 2003). In addition, it has also been shown that diazinon caused an increase in lipid peroxidation in rat erythrocytes (Sutcu et al., 2007). Treatment of rats with diazinon eventuated in decreased renal antioxidants and enhanced lipid peroxidation with concomitant renal damage, which are involved in the diazinon-induced renal oxidative stress and toxicity (Dawood Shah and Iqbal, 2010).

It has been shown that antioxidant substances protect cells against deleterious effects of environmental agents. In recent years, some agents with antioxidant effects (e.g. vitamins C and E, N-Acetylcysteine and zinc) have been used to decrease cellular oxidative stress, and thus cellular damage, in cases of organophosphate poisoning (El-Shenawy et al., 2010; Sameeh et al., 2009; Sutcu et al., 2007; Ogutcu et al., 2006; Cankayali, et al., 2005). tert-Butylhydroquinone (TBHQ) is one of the few antioxidants permitted for use in foods. TBHQ has been shown to induce nuclear translocation of Nrf2 that regulates antioxidant genes as an adaptive response to oxidative stress (Tasset et al., 2010).

Little attention has been given to the chronic low dose effects of pesticides, which may not have clinically recognizable symptoms but could produce subtle cumulative metabolic effects that eventually affect the overall health of an animal. In addition, there is no study about the ameliorative effect of TBHQ on oxidative stress induced by diazinon. Therefore, the present study was undertaken to evaluate the possible protective effect of TBHQ on oxidative stress and antioxidant status after 7 weeks exposure to sub-lethal dose of diazinon.
in rats.

Figure 1. Effects of diazinon and TBHQ on the MDA level in liver and kidney of rats. Values are expressed as mean ± SD. Means lacking a common lowercase letter differ ($p<0.05$).

Materials and methods

**Chemicals**

TBHQ, diazinon, tris, potassium chloride (KCl), thiobarbituric acid (TBA), pyridine, n-butanol, sodium hydroxide (NaOH), 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB), ethylenediaminetetraacetic acid (EDTA), sodium acetate trihydrate, glacial acetic acid, 2,4,6-tripyridyl-s-triazine (TPTZ), hydrochloric acid (HCl), FeCl$_3$, 6H$_2$O and FeSO$_4$.7H$_2$O of technical grade used in this study were supplied by Sigma-Aldrich (Germany) or Merk (Germany) companies.

**Animals**

Male Wistar-rats (weighing approximately 230–250 g) were purchased from the Razi Vaccine and Serum Research institute, Mashhad, Iran. All these animals were acclimatized for one week before the onset of experiment. The animals were housed in plastic (polypropylene) cages using paddy husk bedding at room temperature (25 ± 1°C) in a 12-hour light/dark cycle with 50 ± 5% humidity. The animals had free access to commercial pellet diet (Javaneh Khorasan, Mashhad, Iran) and water ad libitum. The experiment was approved by the Animal Welfare Committee of the School of the Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran.

**Experimental protocol**

Twenty eight adult male Wistar-rats divided randomly into four groups having seven animals in each. The compounds were administrated in the morning (between 9:00 and 11:00 AM) to non-fasted rats. All rats were treated for 7 weeks.

- **Group 1**: control group
  The control group received corn oil (vehicle of diazinon and TBHQ) through gavages once a day.

- **Group 2**: diazinon-treated group
  Diazinon at a dose of 10 mg/kg BW/day in corn oil was given through gavage to rats once a day. The selection of dose regimen was based on previously published studies which indicate substantial alterations in many of the biochemical parameters at this dose (Dawood Shah and Iqbal, 2010; Kalender et al., 2005).

- **Group 3**: TBHQ treated group
  The compound received corn oil (vehicle of diazinon and TBHQ) through gavages once a day.

- **Group 4**: diazinon-TBHQ group
  Diazinon at a dose of 10 mg/kg BW/day in corn oil was given through gavage to rats once a day. The selection of dose regimen was based on previously published studies which indicate substantial alterations in many of the biochemical parameters at this dose (Dawood Shah and Iqbal, 2010; Kalender et al., 2005).
previously published studies which indicate anti-oxidative effects of TBHQ at this level (0.028 g/kg of diet; Nishizono et al., 2000). For this purpose, the food intake of rats was measured during the acclimatization period. Pre-weighed food was provided in standard stainless steel hoppers. After 24 h, the amount of food remaining, including any on the bottom of the cages or any that had spilled onto plastic sheets placed under each cage was recorded. Intake was calculated as the weight (in grams) of food provided minus that recovered. Based on recommendations for the most effective dose of TBHQ (0.028 g/kg of diet) and registered food intake, the final concentration of TBHQ was calculated and was given through gavage to rats once a day.

Group 4: TBHQ + diazinon-treated group

TBHQ and diazinon (at above-mentioned doses) were administered orally via gavage needle.

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Tissue preparation

The animals were euthanized by CO₂ 24 h after the last dose of diazinon or TBHQ. Liver and kidney tissues of these animals were taken quickly, cleaned free of extraneous material and perfused immediately with sodium phosphate buffer (pH 7.4). Tissue samples were minced, cut into small pieces and then dried on a filter paper and homogenized (10% w/v) in ice-cold 1.15% KCl-0.01 M sodium, potassium phosphate buffer (pH 7.4) by “Silent crusher M” type homogenizer (Heidolph Instruments GmbH & Co. KG, Schwabach, Germany). The homogenate was centrifuged at 18,000g for 20 min at 4°C, and the resultant supernatant was used for the determination of oxidative stress markers.

Lipid peroxidation assay

The extent of lipid peroxidation was estimated as the concentration of thiobarbituric acid reactive product malondialdehyde (MDA) by using the method of Placer et al. (1966). The reaction mixture consisted of 0.2 ml of homogenized tissue, 1.3 ml of 0.2 M Tris–0.16 M KCl buffer (pH 7.4) and 1.5 ml of thiobarbituric acid reagent. The mixture was heated in a boiling water bath for 10 min. After cooling, 3 ml of pyridine/n-butanol (3:1, v/v) and 1 ml of 1 N sodium hydroxide were added and mixed by vigorous shaking. A blank
was run simultaneously by incorporating 0.2 ml distilled water instead of the homogenized tissue. The absorbance of the test sample was read at 548 nm. The nmol of MDA per ml was calculated using $1.56 \times 10^5$ as extinction coefficient.

**Total thiol groups assay**

Total thiol groups of homogenized tissues were measured spectrophotometrically at 412 nm using DTNB as the reagent (Hu and Dillard, 1994). After adding tris buffer to homogenized tissue, first absorbance was read at 412 nm (A1). Then DTNB was added and second absorbance at 412 nm was done (A2). The concentration of total thiol groups was calculated and expressed as mmol/l.

**Ferric reducing antioxidant power (FRAP) assay**

The total antioxidant capacity of the homogenized tissues was measured using FRAP assay, which depends upon the reduction of ferric tripyridyltriazine (Fe(III)-TPTZ) complex to the ferrous tripyridyltriazine (Fe(II)-TPTZ) by a reductant at low pH. (Fe(II)-TPTZ has an intensive blue color and can be monitored at 593 nm (Benzie and Strain, 1996).

**Statistical analysis**

Statistical analysis was conducted using SPSS for windows (release 16, SPSS Inc., Chicago, IL) with a $p$ value of <0.05 as statistically significant. Data were expressed as mean±standard deviation (SD). One way ANOVA was used to compare means among the different groups. Following analysis of variance, significant between-group differences were detected by the bonferroni test.

**Results**

A significant increase in MDA levels ($p<0.05$) in the kidney tissue was evident in the diazinon group, when compared to the control group. However, no statistically significant differences were detected for liver MDA between diazinon and control groups. TBHQ alone or associated with diazinon, did not reduce lipid peroxidation in the evaluated tissues and no significant differences were observed for MDA level between TBHQ and
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Treatment with diazinon did not induce any significant change in the thiol group and FRAP levels of liver and kidney tissues. TBHQ alone or associated with diazinon, significantly increased thiol groups. Rats of the TBHQ and diazinon-TBHQ groups presented a significant increase in thiol groups ($p<0.05$), when compared to control and diazinon groups (fig.2). Co-administration of TBHQ with diazinon increased FRAP level in the liver and kidney tissues ($p<0.05$), when compared with the diazinon group (fig.3).

**Discussion**

Extensive application of OPs is usually accompanied with serious problems of pollution and health hazards. OPs act as pro-oxidants and elicit oxidative effects in multiple organs (Limon-Pacheco and Gonsebatt, 2009). Bagchi *et al.* (1995) found that OPs may induce in vitro and in vivo generation of ROS, such as hydrogen peroxide, superoxide and the hydroxyl radical. Oxidative stress has been described in acute and chronic exposure to OPs in both animals and humans (El-Shenawy *et al.*, 2010; Agrawal and Sharma, 2010; Eduardo *et al.*, 2006; Mashali *et al.*, 2005; Kovacic, 2003; Zhou, *et al.*, 2002; Banerjee *et al.*, 2001). The present study was designed to elucidate the potential antioxidant effects of TBHQ, which is used as a food-additive antioxidant for human.

In the present study, diazinon promoted lipid peroxidation in the kidney tissue of rats treated with 10 mg/kg of diazinon for 7 weeks. Lipid peroxidation causes profound alterations in the structure and functions of the cell membrane including decreased membrane fluidity and increased membrane permeability (Ojha *et al.*, 2011). Lipid peroxidation results in the disarrangement and ultimately, disruption of cell membranes, which leads to cell death (Oruc and Usta, 2007). It has been shown that OPs caused increase of lipid peroxidation through their interference with membrane dependent processes (Kalender *et al.*, 2007, 2010; Elhalwagy *et al.*, 2008; Uzun *et al.*, 2010; El-Demerdash, 2011). In consistent with the present study, Dawood Shah and Iqbal (2010) reported that treatment of rats with diazinon significantly enhanced renal lipid peroxidation in a dose-dependent manner, reflecting the formation of ROS in rat kidney. At the lower dose of diazinon 10 mg/kg body weight, about 1.1-fold increase in lipid peroxidation occurred compared to control. However, at the higher dose of diazinon 30 mg/kg body weight, the increase in lipid peroxidation was around 1.3-fold (Dawood Shah and Iqbal, 2010). In addition, enhanced lipid peroxidation has been observed following diazinon administration in erythrocytes (Altuntas, *et al.*, 2004), liver (Amirkabirian *et al.*, 2007) brain (Jafari *et al.*, 2012), spleen (Jafari *et al.*, 2012) and pancreas (Gkalp *et al.*, 2005) of rats. High lipid peroxidation may be due to oxidation of molecular oxygen to produce superoxide radicals. This reaction is also the source of H$_2$O$_2$, which causes the production of MDA by initiating the peroxidation of unsaturated fatty acids in the membrane (Uner *et al.*, 2001). The difference of results in different studies is caused by the dose, exposure time, and administration route of toxin. The observed differences in diazinon-induced lipid peroxidation among various tissues may depend on several factors such as oxygen consumption, metabolic activity rate, susceptibility to oxidants and many more (Jafari *et al.*, 2012).

TBHQ, one of the most powerful synthetic antioxidants, is recommended by American Institute of Nutrition (AIN) as a supplement to the rodent diet (Philip *et al.*, 1993). Anti-oxidative effect of TBHQ has been reported in experimental studies. TBHQ reduced the level of lipid peroxidation in mice with traumatic brain injury (Lu *et al.*, 2014). It protects the living animal and cell lines against acute
toxicity and oxidative insult (Lu et al., 2014). TBHQ is able to induce the nuclear translocation of transcription factor NF-E2-related factor 2 (Nrf2), which in turn regulates the expression of vitagenes codifying for cytoprotective phase 2 antioxidant proteins, such as glutathione- S-transferase, NAD(P)H quinone oxidoreductase and heme-oxygenase-1 (Tasset et al., 2010). In the present study, Co-administration of TBHQ with diazinon significantly increased total thiol groups and FRAP levels in liver and kidney tissues. In agreement with the present study, Lu et al. (2014) reported that pretreatment with TBHQ significantly increased antioxidant activity in mice with traumatic brain injury. Although TBHQ increased antioxidant levels in rats receiving diazinon, it could not attenuate lipid peroxidation in them.

While diazinon administration showed no significant alterations on antioxidant levels, combined TBHQ and diazinon administration increased antioxidant level in liver and kidney tissues. The observed results could be explained by increased challenge with free radicals and oxidants in the diazinon-received rats. In the TBHQ + diazinon group, elevated free radical generation could be encountered with increased antioxidant capacity via TBHQ administration. The elevated levels of measured antioxidants are due to the adaptive response to the generated free radicals. The increase in antioxidants may be explained by the fact that oxidants activate gene expression through antioxidant responsive elements (Rushmore et al., 1991). However, the total antioxidant defense mechanism was not able to protect the tissues from oxidative damage caused by diazinon, as evidenced by lipid peroxidation.

The present study is the first investigation in which protective effects of TBHQ against diazinon-induced oxidative stress was evaluated. Several limitations of the present study should be considered when interpreting the results. The used amount of TBHQ in the present study was selected based on recommendations in the AIN-93G formula (Philip et al., 1993). Higher amounts of TBHQ might be more effective in attenuation of diazinon-induced lipid peroxidation.

**Acknowledgements**

This study was supported by research fund of Ferdowsi University of Mashhad (project no. 3/29214). The authors wish to thank technicians who kindly helped us for sample collection of this study.

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اثرات محافظتی ترت بوتیل هیدرو کوئینون (TBHQ) در مقایسه اکسیدانی ناشی از دیازینون در رت

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چکیده

با توجه به نقش استرس اکسیدانی در ایجاد اسیدهای ترکیبی سومی اکسیدونفرسی در مطالعه حاضر اثرات مسمومیت مزمن با دیازینون بر شاخص‌های استرس اکسیدانی بافت‌های کبدی و کلیه رت و استار مورد ارزیابی قرار گرفت. به علاوه اثرات محافظتی ترت بوتیل هیدرو کوئینون (TBHQ) به عنوان یک آنتی اکسیدان غذایی قوی در جلوگیری از بروز آسیب‌های اکسیدانی ناشی از دیازینون در این بات‌ها بررسی گردید. گروه های مورد مطالعه شامل 4 گروه هر کدام به تعداد 7 رت بود؛ گروه کنترل (دریافت کننده روغن زرد) گروه دیازینون (10 میلی گرم به ازای هر کیلوگرم وزن بدن حیوان در روز)، گروه TBHQ (0/28/0، گرم به ازای هر کیلوگرم ماس حیوان، حل شده در روغن زرد) و گروه TBHQ دیازینون- TBHQ (سم و آنتی اکسیدان با دوزهای ذکر شده)، خواص آنتی اکسیدان‌ها و میزان تولید تکثیر تجویز همزمان با دیازینون، مقادیر فرمول ترکیبی TBHQ در حیاتی کبدی و کلیه با فرمول ترکیبی TBHQ و میزان تولید تکثیر تجویز همزمان با دیازینون، مقادیر فرمول ترکیبی TBHQ در حیاتی کبدی و کلیه

واژگان کلیدی: دیازینون، استرس اکسیدانی، ترت بوتیل هیدرو کوئینون، رت