The Journal of Toxicological Sciences (J. Toxicol. Sci.)
Vol.36, No.3, 285-296, 2011

Original Article

Hepatoprotective effects of vitamin E/selenium against malathion-induced injuries on the antioxidant status and apoptosis-related gene expression in rats

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(Received February 21, 2011; Accepted March 29, 2011)

ABSTRACT — The present study is undertaken to evaluate the protective effect of vitamin E (α-tocopherol) and selenium (Se) against malathion (MTN)-induced oxidative stress and hepatic injuries in experimental rats. Male rats were randomly divided into eight groups comprised of 10 rats each. The 1st group served as a negative control (C_N), whereas the 2nd was supplemented with a combination of α-tocopherol (100 mg kg^{-1} body weight, b.w.)/Se (0.1 mg kg^{-1} bw). The 3rd, 4th and 5th groups were respectively administered with increasing doses of MTN equivalent to $_{1/50}LD_{50}$ ($M_{1/50}$), $_{1/25}LD_{50}$ ($M_{1/25}$) and $_{1/10}LD_{50}$ ($M_{1/10}$), respectively. The 6th, 7th and 8th groups were administered the same doses of MTN as in the 3rd, 4th and 5th groups with a concomitant supplementation with α-tocopherol/Se. Subchronic exposure of rats to MTN for 45 days resulted in statistical dose-dependent decrease in acetylcholinestrase (AChE) activity, increase in oxidative stress marker lipid peroxidation (LPO) and reduction in reduced glutathione (GSH) level. Moreover, the levels of glutathione persoxidase (GPx), superoxide dismutase (SOD) and catalase (CAT) were significantly decline in response to MTN exposure in a dose-dependent fashion. Furthermore, histopathological studies of liver in the rats which received MTN exhibited, moderate to severe degenerative and necrotic changes in the hepatocytes. Notably, the administration of α-tocopherol/Se protected the liver of rats exposed to MTN as evidenced by the appearance of normal histological structures, significant attenuation of the decline in all antioxidant enzymes tested (i.e. GPx, SOD and CAT), significant recovery in the GSH level and statistical reduction in LPO, as compared to the experimental rat. The effect of α-tocopherol/Se supplementation on transcriptional activity of three key stress and apoptosis-related genes (i.e., *Tp53*, *CASP3* and *CASP9*), in response to MTN exposure in rats, was investigated. Results revealed a significant concentration-dependent up-regulation in the level of expression for the three genes examined, in response to MTN exposure, compared with the control. Interestingly, the supplementation of MTN-treated rats with α-tocopherol/Se modulates the observed significant dose-dependent up-regulation in the level of expression for three selected genes, indicative of an interfering role in the signaling transduction process of MTN-mediated poisoning. Taken together, these data suggest that the administration of α-tocopherol/Se may partially protect against MTN-induced hepatic oxidative stress and injuries.

Key words: Malathion, Antioxidant enzymes, Oxidative damage, α-Tocopherol, Selenium, Apoptosis-related genes, Real-time PCR

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INTRODUCTION

Organophosphorous insesticides (OPIs) have extensively been used since the mid 1940s and represent a major class of chemical compounds most commonly applied in agricultural pest control programs. They are recognized as a serious source of environmental pollution and health hazard due to their ability to exert significant adverse toxicity in non-target species including human, through the inhibition of acetylcholinestrase (AChE). This leads to the accumulation of acetylcholine and subsequent activation of cholinergic muscarinic and nicotinic receptors (Savolainen, 2001; Costa, 2006; Rezg *et al*., 2008). Moreover, OPIs could induce oxidative stress, which might play a crucial role in its induced poisoning, supported by *in vivo* animal and human studies (Monteiro *et al*., 2006; Zhou *et al*., 2002) and *in vitro* studies (Gultekin *et al*., 2000) over the past several years.

Malathion [*O,O*-dimethyl-S-(1,2-dicarcethoxyethyl) phosphorodithioate] is an OPI that is extensively used to control a wide range of sucking and chewing pests of field crops, fruits, and vegetables. Once malathion (MTN) is introduced into the environment, it may be activated by atmospheric photooxidation, or degraded by hydrolysis, or biodegradation mediated by microorganisms found in most sediment, soils, and water. Malaoxon, the oxon generated from MTN, is more toxic than MTN and is formed by the oxidation of MTN and may also be present as an impurity in the parent compound. Structurally, MTN has similarities with naturally occurring compounds, and their primary target of action in insects is the nervous system (Taylor, 1990; Klaasen, 1990).

The toxicity of MTN and other OPIs is ascribed, at least in part, to the generation of reactive oxygen species (ROS), leading to lipid peroxidation (LPO). Moreover, malathion is a lipophilic substance; it may enhance LPO by directly interacting with the cellular plasma membrane (Banerjee, 1999).The liver is among the primary targets for MTN toxicity and is considered as the major active metabolizing organ which mediates the bio-activation of thiono-organophosphates (Yang *et al*., 2000).

Oxidative stress occurs as a consequence of imbalance between cellular natural antioxidative defences and prooxidant state (e.g. generated by OPIs toxicity) culminating into the production of potentially destructive ROS. This ROS-mediated damage includes alterations of cellular macromolecules such as membrane lipids, DNA, and/or proteins. The damage may alter cell function through changes in intracellular calcium or intracellular pH, and eventually can lead to cell death (Kehrer *et al*., 1990; Pham-Huy *et al*., 2008). Generally, the accumulation of ROS can involve cytotoxicity and enormous damage to DNA, as well causing an alteration in the expression of genes including those of apoptosis-related genes. The application of gene expression profiling in toxicological studies, has the potential to allow deeper understanding of the mechanisms of toxicity (Kramer and Kolaja, 2002; Ulrich and Friend, 2002; Waters *et al*., 2010).

The antioxidant enzymes, superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) are the main endogenous enzymatic defence system and act in concert with a panoply of non-enzymatic antioxidants, such as reduced glutathione (GSH), vitamin E (α-tocopherol) and selenium (Se), for the conversion of ROS to harmless metabolites, as well as to protect and restore normal cellular metabolism and functions (Halliwell and Gutteridge, 1999; Bebe and Panemangalore, 2003). Thus, α-tocopherol is an important component in human diet and considered the most effective lipid-soluble antioxidant found in the biological system that scavenges ROS, thereby preventing LPO and the initiation of oxidative tissue damage (Evstigneeva *et al*., 1998; Verma *et al*., 2007; El-Shenawy *et al*., 2010). This exquisite protective role emanates from its efficient breaking property for the ROS chain reactions by allowing ROS to abstract a hydrogen atom from the antioxidant molecule rather than from polyunsaturated fatty acids, resulting in the formation of an unreactive α-tocopheroxyl radical (Sies and Murphy, 1991; Packer, 1991). Moreover, α-tocopherol has been shown to affect bone structure by increasing bone trabecular formation (Xu *et al*., 1995), preventing the impairment of bone calcification caused by the exposure to oxidizing agents (Yee and Ima-Nirwana, 1998). To date, driven by the frequent exposure-related health problems, tremendous research interests have been focused on evaluating the protective effects of α-tocopherol on pesticide-induced oxidative damage (Jalili *et al*., 2007; El-Gharieb *et al*., 2010; El-Shenawy *et al*., 2010; Kalender *et al*., 2010). The trace element Se is an essential nutrient which has been reported to counteract ROS and protect the structure and function of proteins, DNA and chromosomes against oxidation injury (Thomson, 2004; Akhtar *et al*., 2009). The synergistic effect of α-tocopherol and Se has proven powerful in reducing storage and toxicity of ROS (Schwenke and Behr, 1998; Aslam *et al*., 2010). Se plays an antioxidant role of fundamental importance to human health. Moreover, Se is an integral part of many proteins with catalytic and structural functions. Most importantly, Se is a constituent of the cytosolic enzyme glutathione peroxidase and facilitates the action of α-tocopherol in reducing peroxy radicals. In chickens, absorption of α-tocopherol

is impaired by severe selenium deficiency, and selenium alleviates α-tocopherol deficiencies by allowing higher α-tocopherol absorption rates (Machlin, 1991).

In this context, studies on α-tocopherol and Se are promising, mainly due to their antiradical activity, as they could provide an important dietary source of antioxidants. Therefore, the present study was undertaken to investigate the protective effect of α-tocopherol/Se in ameliorating oxidative damage and hepatic injuries in male rats administered with subchronic doses of malathion. Moreover, the impact of α-tocopherol/Se co-administration on malathion-mediated expression level of selected apoptosis-related marker genes was also examined (i.e. *Tp53*, *Caspase-3*, *Caspase-9*), *via* quantitative reverse-transcriptase PCR (qRT-PCR).

MATERIALS AND METHODS

Chemicals

All fine chemicals used in this study were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals, including Vitamins E ($_{DL}$-α-tocopherol acetate), were supplied by Merck were analytical grade or better from E. Merck (Darmstadt, Germany). Commercially available malathion (agrothion 57% EC 500 g l^{-1}) was used in this study.

Animals and experimental design

Eighty male Wister albino rats, weighing 200-250 g, were obtained from the animal house, Collage of Medicine, King Saud University. Animals were housed under standard lighting and relative humidity (50 ± 15%) conditions in a temperature-controlled room (25 ± 2ºC). Animals were allowed to acclimatize to the laboratory environment for 10 days, and were allowed free access to standard dry pellet diet and water *ad libitum*. All animal experiments reported here complied with the ethics and regulations of the Animal Care and Use Committee of King Saud University, Saudi Arabia. Subsequently, rats were randomly separated in 8 polypropylene cages of ten individuals each and were administered orally 0.5 ml of solution *via* gavage. Rats from the 1st (control) group orally received a daily dose of 0.5 ml corn oil for a period of 45 days. Animals of the 2nd, 3rd and 4th experimental groups were given daily doses of MTN at the level of $_{1/50}$ LD_{50} (27.5 mg kg^{-1} body weight, bw), $_{1/25}$ LD_{50} (55 mg kg^{-1} bw), $_{1/10}$ LD_{50} (137.5 mg kg^{-1} bw), respectively, diluted in 0.5 ml corn oil. The LD_{50} value employed was 1,375 mg kg^{-1} bw, according to Gaines (1960). The 5th, 6th and 7th groups were administered daily Selenium (0.1 mg kg^{-1} bw) + α-tocopherol (100 mg kg^{-1} bw) combination concomitantly with malathion doses of $_{1/50}$ LD_{50}, $_{1/25}$ LD_{50} and $_{1/10}$ LD_{50}, respectively, for 45 days. Animals of the 8th group solely received a daily combination of selenium + vitamin E for the same period of time. The substances were administrated in the morning (between 09:00 AM and 10:00 AM) to non-fasted animals. Selenium and vitamin E were dissolved in water and corn oil, respectively. Selenium (0.1 mg kg^{-1} bw) and vit E (100 mg kg^{-1} bw) doses were chosen on the basis of previously published data (Stajn *et al*., 1997; Chowdhury *et al*., 1987; El-Demerdash *et al*., 2004; Yousef *et al*., 2006; Agar *et al*., 2003; Patra *et al*., 2001). At the end of experimentation, animals were sacrificed under anesthesia and the liver were removed, weighted and treated for biochemical measurements of antioxidant enzymes and parameters.

Tissue preparation

Liver was removed from rats under ether anaesthesia after 45 days of treatment and washed with cold saline buffer. For RNA isolation, washed tissues were immediately snap-frozen in liquid N_2 and stored at -80°C until further use. To obtain the enzymatic extract, approx. 1 g of fresh liver tissue was homogenized in 10 ml of buffer solution of phosphate-buffered saline (PBS) 1:2 (w/v; 1 g tissue with 10 ml PBS, pH 7.4). Homogenates were centrifuged at 8,000 rpm for 15 min at 4°C, and the resultant supernatant was used for the biochemical determinations.

Protein estimation

The protein contents of various samples were determined according to the method of Bradford (1976) by using bovine serum albumin as a standard.

Determination of acetylcholinesterase

Acetylcholinestrase (AChE, EC 3.1.1.7) activity was assayed as previously described (Ellman *et al*., 1961).

Determination of superoxide dismutase

Superoxide dismutase (CuZnSOD; EC 1.15.1.1) activity in liver was assayed spectrophotometrically, using nitroblue tetrazolium as the indicator reagent, according to the protocol of Kakkar *et al*. (1984).

Determination of catalase

Catalase (CAT, EC 1.11.1.6) activity in liver was measured using hydrogen peroxide (H_2O_2) as substrate as previously described (Aebi, 1983).

Determination of glutathione peroxidase

Glutathione peroxidase (GPx, EC 1.11.1.9) activity was measured using Paglia and Valentine's method (1967).

Determination of thiobarbituric acid reactive substances (TBARS)

The extent of LPO in liver homogenate was estimated as the concentration of malondialdehyde (MDA), which is the end product of lipid peroxidation, and reacts with thiobarbituric acid (TBA) as a TBA reactive substance (TBARS) according to Ohkawa *et al*. (1979).

Determination of reduced GSH

Liver GSH content was estimated using a colorimetric technique, based on the development of a yellow colour when DTNB [(5,5 dithiobis-(2-nitrobenzoic acid)] is added to compounds containing sulfhydryl groups, according the method described by Sedlák and Lindsay (1968).

Histopathological examination

Liver samples were dissected and fixed in 10% neutral buffered formalin, dehydrated in ascending grades of alcohol and imbedded in paraffin wax. Next, Paraffin sections (5 µm thick) were stained for routine histological study using haematoxylin and eosin for histological evaluation under light microscope. The sections were viewed and photographed by using an Olympus light microscope (Olympus BX51, Tokyo, Japan) with an attached photograph machine (Olympus E-330, Olympus Optical Co. Ltd.). Five slides were prepared from each liver and sections were evaluated for liver injury.

RNA isolation

Frozen liver sections were grinded to a fine powder in a Mixer Mill MM 200 (Retsch GmbH and Co. KG, Haan, Germany) using precooled stainless steel balls. Subsequently, total RNA was isolated with iPrep™ PureLink™ Total RNA Kit (Invitrogen GmbH, Darmstadt Germany) using the automated iPrep™ Purification Instrument (Invitrogen GmbH), including a DNase digestion step according to the manufacturer's instructions. The final product was quality controlled by gel analysis using RNA HighSens Analysis Chips on an Experion System (Bio-Rad Laboratories GmbH, Munich, Germany.) Only samples with a peak area ratio ≥ 2.0 of 28S to 18S rRNA were used. RNA concentrations were determined with a NanoDrop 8000 Spectrophotometer (Thermo Scientific, Bonn, Gemrany).

Quantitative reverse-transcriptase PCR (qRT-PCR) technique

qRT-PCR analysis of the expression of apoptosis-related genes was conducted as previously described (Al-Roba *et al*., 2011). The first-strand cDNA synthesis was performed with 1 µg of total RNA using 100 ng of oligo-p$(dT)_{12-18}$ primer and MLV Reverse Transcriptase using Ready-To-Go RT-PCR Beads (GE Healthcare GmbH, Germany), according to the manufacturer's recommendations. Expression levels were normalized to *GAPDH* gene expression, which was used as an internal housekeeping control. Real-time quantification was performed in the LightCycler® 480 Instrument with 96-well plate (Roche Diagnostics GmbH, Mannheim, Germany) using the qPCR GreenMaster based on EvaGreen Fluorescent DNA Stain (Jena Bioscience GmbH, Germany). PCR mixtures (final volume of 20 µl) contained 10 µl of qPCR GreenMaster (Jena Bioscience), 5 µl of a 10^{-2} dilution of the cDNA and 300 nM of each primer (Table 1). The cycling conditions included an initial heat-denaturing step at 95°C (Ramp Rate 4.4°C/sec) for 5 min, 50 cycles at 95°C for 20 sec (Ramp Rate 4.4 °C/sec), annealing at 60°C for all the primers for 15 sec (Ramp Rate 2.2°C/sec), and product elongation and signal acquisition (single mode) at 72°C for 15 sec (Ramp Rate 4.4°C/sec). Following amplification, the melting curves were determined in a three-segment cycle of 95°C for 0 sec, 65°C for 15 sec, and 95°C for 0 sec at the continuous acquisition mode. The temperature transition rates

Table 1. List of primers used for qRT-PCR expression analysis of apoptosis-related genes of rat

Target Gene	Forward (F) and reverse (R) Primers (5'→3')	GenBank Accession no.	$T_{(a)}$
Caspase-3	F: 5'-CAGAGCTGGACTGCGGTATTGA-3 R: 5'-AGCATGGCGCAAAGTGACTG-3'	NM_012922	60°C
Caspase-9	F: 5'-AGCCAGATGCTGTCCCATAC-3' R:5'-CAGGAGACAAAACCTGGGAA-3'	AF262319	60°C
Tp53	F: 5'-GTCGGCTCCGACTATACCACTATC-3' R:5'-CTCTCTTTGCACTCCCTGGGGG-3'	NM_030989	60°C
GAPDH	F: 5'-GCTGCCTTCTCTTGTGACAAAGT-3' R: 5'-CTCAGCCTTGACTGTGCCATT-3'	AF106860	60°C

were set at 20°C/sec except for segment three of the melting curve analysis where it was set to 0.1°C/sec. Water was used as the template for negative control amplifications included with each PCR run. Serial dilutions of each cDNA (10^{-1}-10^{-6}) were used to generate a quantitative PCR standard curve to calculate the corresponding PCR efficiencies. Results reported were obtained from three biological replicates and PCR runs were repeated twice.

qRT-PCR fold change calculations

Data were analyzed using the Roche LightCycler® 480 software Version 3.5. Crossing point (C_p) was calculated by the Second Derivate Maximum Method. The amount of the target mRNA was examined and normalized to the GAPDH gene mRNA. The relative expression level (Fold change) was analyzed using the $2^{-\Delta\Delta Cp}$ method (Livak and Schmittgen, 2001). Briefly, the relative level of each mRNA normalized to the *GAPDH* gene was calculated using the following equation: Fold change = $2^{\text{Cp target (control)} - \text{Cp target (treatment)}}/2^{\text{Cp GAPDH (control)} - \text{Cp GAPDH (treatment)}}$. The relative gene expression data were subjected to Student's *t*-test in order to identify significant differences between pesticide-treated samples compared to untreated control. Treatments were considered statistically significant when at $P < 0.05$. Statistical analyses were undertaken using MINITAB software (MINITAB, State College, PA, v. 13.1, 2001).

Statistical analysis

The calculations and statistical analysis were carried out using the Statistical Package for Social Sciences (SPSS) for Windows version 17.0. All data were represented as mean ± standard deviation (S.D.). Data were subjected to one-way analysis of variance (ANOVA) followed by Bonferroni's post-hoc test for multiple comparisons of the means between treatments and the negative control. The correction method was applied and statistical probabilities $P < 0.05$ were considered to be significant.

RESULTS

Effect of α-tocopherol/Se on AChE activity and oxidative stress markers

Liver acetylcholinestrase (AChE) activity is depicted in Fig. 1A. The AChE activity exhibited a significant ($P < 0.05$) dose-dependent inhibition in MTN-treated rats as a function of increasing concentration. Values of AChE inhibition were by 24.8%, 40% and 62.2% in experimental groups treated with $_{1/50}LD_{50}$ ($M_{1/50}$), $_{1/25}LD_{50}$ ($M_{1/25}$) and $_{1/10}LD_{50}$ ($M_{1/10}$), as compared to the negative control group (C_N), respectively (Fig. 1A). The α-tocopherol/

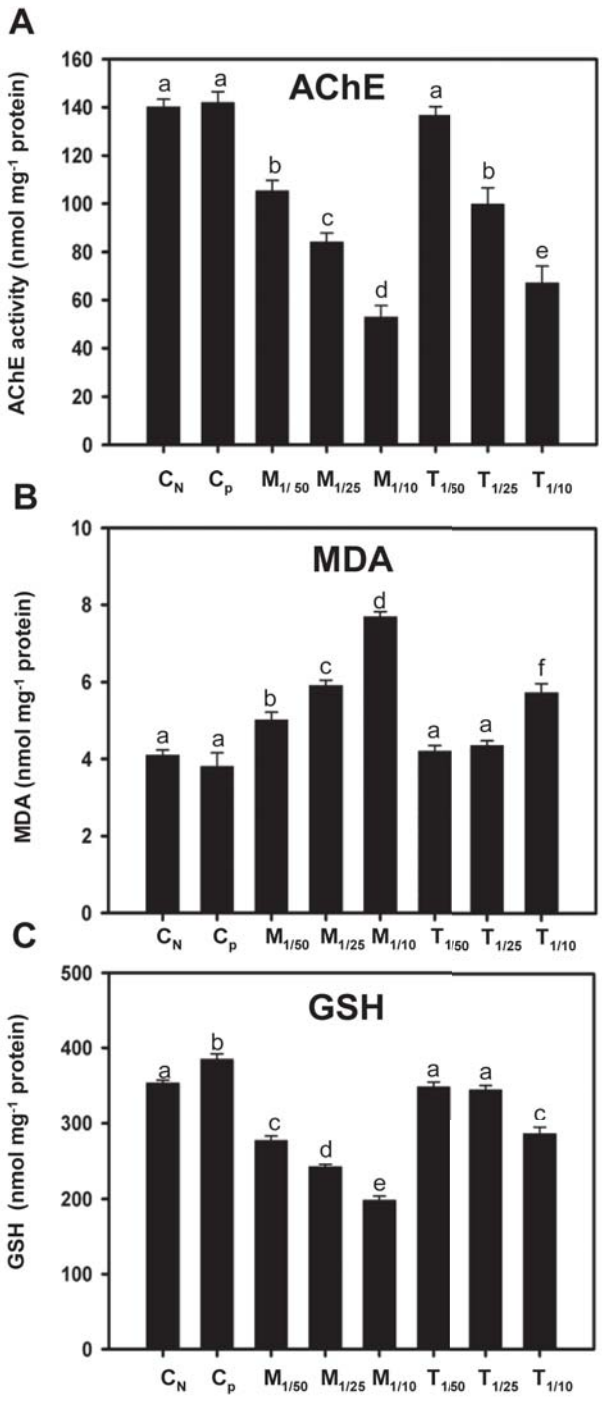

Fig. 1. Effect of α-tocopherol/Se on oxidative stress parameters in liver from negative control and malathion-treated experimental groups. (A) AChE, (B) malondialdehyde (MDA), and (C) reduced glutathione (GSH). Data represent the means ± S.D. (n = 10). Different letters indicate is significantly different mean values at $P < 0.05$.

Se treatment significantly ($P < 0.05$) attenuated the observed MTN-mediated inhibition in AChE activity by 29.8% ($T_{1/50}$), 18.7% ($T_{1/25}$) and% 26.8 ($T_{1/10}$), compared to their respective rat groups which only received MTN. A complete restoration in the activity level of AChE was observed supplemented with α-tocopherol/Se in rats exposed to the lowest MTN dose ($_{1/50}LD_{50}$, $T_{1/50}$), as compared to the C_N group (Fig. 1A). The group of rats which only received the α-tocopherol/Se treatment (C_p, control positive) exerted no apparent impact on the level of AChE enzyme activity compared with the C_N group (Fig. 1A). The MTN administration significantly ($P < 0.05$) increased liver oxidative stress in a dose-dependent manner as indicated by enhanced malondialdehyde (MDA) production (Fig. 1B). This increase in MDA was by 22%, 44% and 87% in $M_{1/50}$, $M_{1/25}$ and $M_{1/10}$ experimental groups, as compared to the C_N group, respectively (Fig. 1B). The *α-tocopherol/Se* treatment significantly ($P < 0.05$) blocked this enhanced MDA production by 16.2% ($T_{1/50}$), 26.2% ($T_{1/25}$) and 25.3% ($T_{1/10}$) compared with experimental rat groups which were only administered with MTN doses of $M_{1/50}$, $M_{1/25}$ and $M_{1/10}$, respectively (Fig. 1B). A comprehensive normalization in MDA levels was evident in rats experimental groups $T_{1/50}$ and$T_{1/25}$ to its level in C_N group. The C_p group exhibited no apparent alterations in MDA activity level (Fig. 1B). Moreover, significant ($P < 0.05$) reductions in liver content of reduced GSH, as a function of increasing MTN dose, were observed as follows: $M_{1/50}$ (21.7%), $M_{1/25}$ (31.6%), $M_{1/10}$ (43.9%), as compared to the C_N group, respectively (Fig. 1C). The supplementation of rats with α-tocopherol/Se significantly ($P < 0.05$) recovered GSH content by rates of 25.9% ($T_{1/50}$), 43% ($T_{1/25}$) and 44.4% ($T_{1/10}$) as compared to experimental rat groups which were only administered with MTN doses of $M_{1/50}$, $M_{1/25}$ and $M_{1/10}$, respectively (Fig. 1C).

Effect of α-tocopherol/Se on the antioxidant status in MTN-treated rats

The results obtained (Figs. 2A-C), indicated that MTN treatment was related with dose-dependent reductions in the activity of key antioxidant enzymes, namely: GPx (Fig. 2A), SOD (Fig. 2B) and CAT (Fig. 2C). Compared with rats in the C_N, significant ($P < 0.05$) dose-dependent reductions in the activity of GPx by 11.8%, 21.2% and 58.8% in rats treated with MTN doses of $_{1/50}LD_{50}$ ($M_{1/50}$), $_{1/25}LD_{50}$ ($M_{1/25}$), $_{1/10}LD_{50}$ ($M_{1/10}$), respectively, were observed (Fig. 2A). Similarly, SOD activity exhibited significant ($P < 0.05$) reductions by 28.8%, 38% and 58.6% in the MTN-treated rat groups $M_{1/50}$, $M_{1/25}$ and $M_{1/10}$, respectively, compared with C_N group

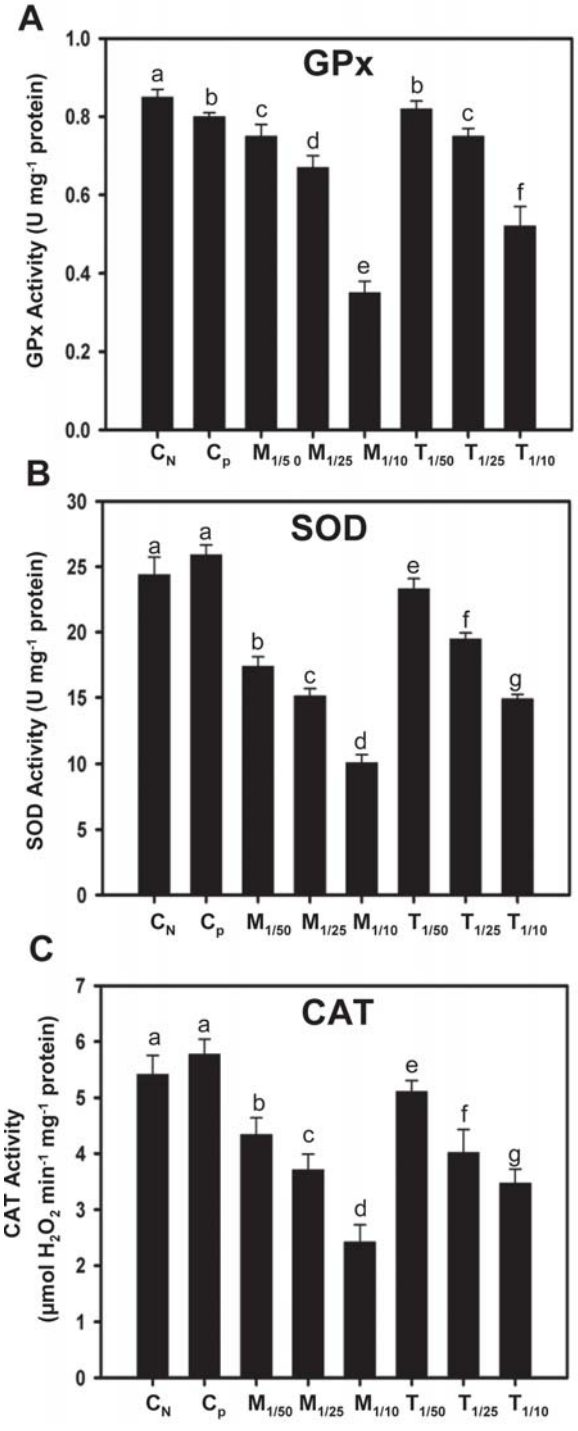

Fig. 2. Effect of α-tocopherol/Se on antioxidant enzyme activity in liver from negative control and malathion-treated experimental groups. (A) GPx, (B) SOD, and (C) CAT. Data represent the means ± S.D. (n = 10). Different letters indicate significantly different mean values at $P < 0.05$.

(Fig. 2B). Again, MTN exposure in the experimental groups $M_{1/50}$, $M_{1/25}$ and $M_{1/10}$ correlated with significant inhibitions in CAT enzymatic activity by 19.8%, 31.4% and 55.3%, when compared with the C_N group (Fig. 2C). In rats supplemented with α-tocopherol/Se a significant ($P < 0.05$) recovery on the activity level of GPx was attained by rates of 9.3% ($T_{1/50}$), 11.9% ($T_{1/25}$) and 48.6% ($T_{1/10}$) as compared to experimental rat groups which were only administered with MTN doses of $M_{1/50}$, $M_{1/25}$ and $M_{1/10}$, respectively (Fig. 2A). Significant ($P < 0.05$) recovery on activity level of SOD was obtained in rats supplemented with α-tocopherol/Se by rates of 34.3% ($T_{1/50}$), 28.9% ($T_{1/25}$) and 47.4% ($T_{1/10}$) as compared to experimental rat groups which were only administered with MTN doses of $M_{1/50}$, $M_{1/25}$ and $M_{1/10}$, respectively (Fig. 2B). The supplementation of rats with α-tocopherol/Se correlated with significant ($P < 0.05$) restoration of CAT enzymatic activity by rates of 17.7% ($T_{1/50}$), 8.4% ($T_{1/25}$) and 43.4% ($T_{1/10}$) as compared to experimental rat groups which were only administered with MTN doses of $M_{1/50}$, $M_{1/25}$ and $M_{1/10}$, respectively (Fig. 2C).

Histopathological findings

Light microscopic observations revealed regular morphology of the liver tissue with well-designed hepatic cells and sinusoids and no pathological alterations in the control group (Fig. 3A). Moreover, the nuclei of the hepatocytes had a normal vesicular structure and the cytoplasm appeared uniform and normal (Fig. 3A). In the 4th group, which had received MTN dose $_{1/10}$ of LD_{50}, severe hepatic lesions with gross degeneration, along with cytoplasmic vacuolation around the nuclei cell death and hyperchromatia, were observed (Fig. 3B). Notably, these histopathological changes in the liver were significantly decreased in the 7th group which had been co-administered the same MTN dose concomitant to α-tocopherol/Se. The supplementation with α-tocopherol/Se provided protection with better arrangement of well-formed polygonal hepatocytes and relatively reduced cytoplasmic vacuolation, in comparison to MTN-treated specimens (Fig. 3C).

Transcriptional activity of apoptosis-related genes

The effect of α-tocopherol/Se supplementation on transcriptional activity of three key stress and apoptosis-related genes (i.e., *Tp53*, *CASP3* and *CASP9*), in response to MTN exposure in rats, was investigated (Figs. 4A-C). Results revealed a significant ($P < 0.05$) concentration-dependent up-regulation in the level of expression for the three genes examined, in response to MTN exposure, compared with the control (Figs. 4A-C). The supplementation with α-tocopherol/Se had a profound modulatory effect on the observed MTN-mediated up-regulation in *Tp53* (Fig. 4A), *CASP3* (Fig. 4A) and *CASP9* (Fig. 4A) genes, leading to their transcriptional inhibition. In response to MTN dose of $_{1/50}$ of LD_{50}, the α-tocopherol/Se supplementation negatively impacted the expression level of all three genes, which was almost completely normalised to its level in the control group.

DISCUSSION

The present study was carried out to examine the protective effect of α-tocopherol/Se treatment on both the antioxidant status and the expression level of selected apoptosis-related genes during MTN-mediated oxidative stress and hepatic injuries in male rats. OPIs exert significant adverse toxicity in non-target species through the inhibition of AChE. In our study, AChE enzyme activity was drastically inhibited by treating rats with three doses of MTN for 45 days, in a dose-dependent manner (Fig. 1A). Inhibition of AChE by OPIs results in the accumulation of acetylcholine at cholinergic synapses, leading to over-stimulation of muscarinic and nicotinic receptors. This "cholinergic syndrome" includes increased sweating and salivation, bronchoconstriction, and bronchial secretion, meiosis, increased gastrointestinal motility, tremors, diarrhea and muscular twitching (Costa, 2006). Hence, it has been adopted as a marker for OPIs-mediated poisoning. Our data clearly suggest that supplementation with α-tocopherol/Se exerted significant reversions in AChE inhibition (Fig. 1A), particularly against the two lowest doses of MTN, indicative of its potential protective effect. The toxicity of OPIs may be due, at least in part, to the formation of ROS, leading to LPO, which is estimated by an increase in the levels of TBARS, assessed by its end product MDA (Hazarika *et al*., 2003; Banerjee *et al*., 1999; Verma and Srivastava, 2001). Moreover, MTN is a lipophilic substance; and it may enhance LPO by directly interacting with the cellular plasma membrane (Hazarika *et al*., 2003). In the present study, remarkable hepatic oxidative damage was observed upon the exposure of rats to three subchronic doses of MTN, as reflected by the dose-dependent increase in MDA production and the substantial inhibition in GSH content (Figs. 1B and C). These findings are in agreement with previous studies which have also demonstrated a decrease in GSH content after exposure to organophosphate and a significant correlation between AChE and GSH (Khan and Kour, 2007; Buyukokuroglu *et al*., 2008). Moreover, the present study indicated that the 45-day MTN treatment in different concentrations induced oxidative

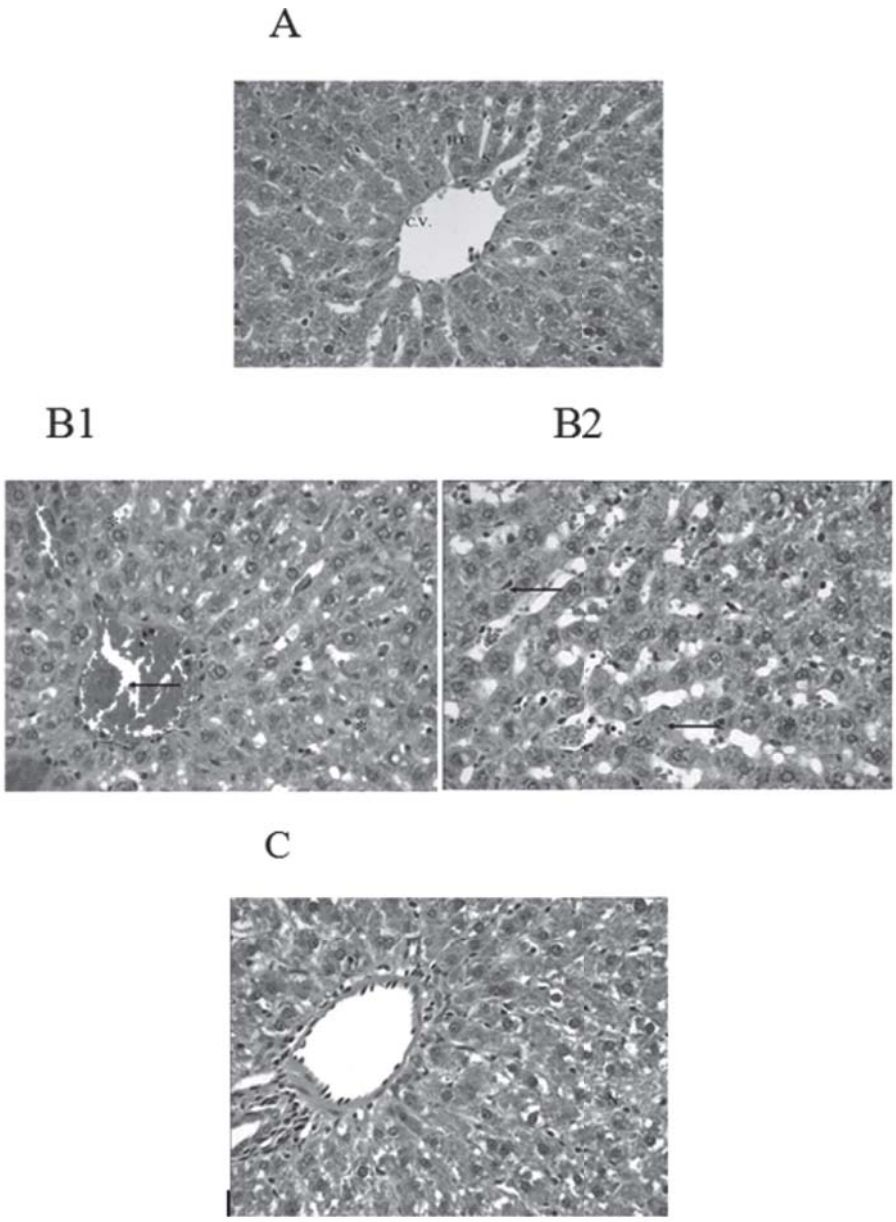

Fig. 3. Paraffin sections stained by haematoxylin and eosin (H&E) for histopathological examination of hepatocytes in untreated control (A), $_{1/10}$ LD_{50} malathion-treated (B1 and B2) and malathion plus α-tocopherol/Se treatment (C). (A) Liver tissue of control showing normal structure, central vein (C.V.), normal arrangement of hepatic cords (H.C.), normal blood sinusoids (S) and hepatocytes (400X); (B1) Liver tissue of malathion treated rats showing hemorrhage in the central vein (←), necrosis; (*) (400X); (B2) Liver tissue of rats treated with malathion $_{1/10}$ LD_{50} showing dilation of blood sinusoids (←), and cell death (*) (400X); and (C) Liver tissue of α-tocopherol/Se-treated rats showing few injuries and cell death (N) (400X).

stress, as demonstrated by compromised dose-dependent enzymatic antioxidant defences (i.e. GPx, SOD, CAT) (Figs. 2A-C). In this context, our data clearly suggest a protective effect of co-supplementation with α-tocopherol/Se evident against MTN-induced poisoning in the liver of rats (Figs. 2A-C). This is supported by our histopathological findings indicating that MTN-mediated liver injuries could be prevented by the treatment with α-tocopherol/Se (Figs. 3A-C). OP insecticides are known to induce various histopathological changes in the liver tissues

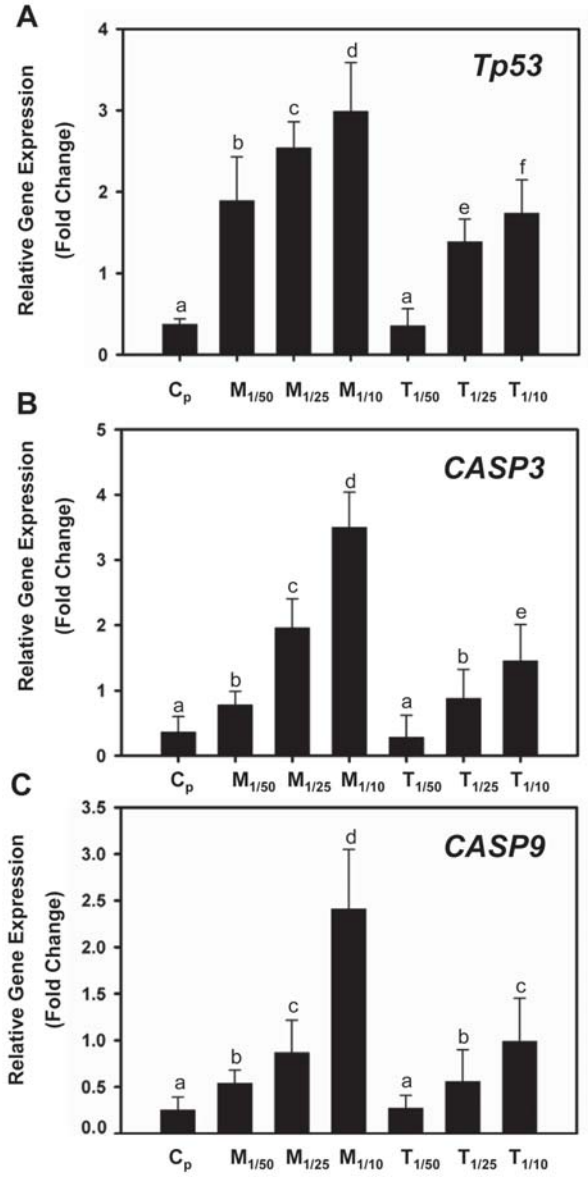

Fig. 4. LightCycler® 480 real-time PCR analysis of apoptosis-related genes in MTN- and α-tocopherol/Se-treated rats. RNA was isolated, reverse transcribed and used for real-time PCR analysis with primers listed in Table 1. Relative expression was determined with the $2^{-\Delta\Delta Cp}$ method using the PCR efficiencies determined with the standard curve included in each run. Each data point represents the results obtained from three independent batches of cDNA made from each stage or tissue. (A) *Tp53* gene expression, (B) *CASP3* gene expression and (C) *CASP9* gene expression. Expression of target genes is normalized to the reference housekeeping gene *GAPDH* and is represented as mean ± S.E. Different letters indicate significantly different mean values at $P < 0.05$ using a paired Student's *t*-test.

(Morowati, 1997; Gokcimen *et al*., 2007; Sayım, 2007; Yehia *et al*., 2007). Conforming to the literature, indeed we found by light microscopic analyses that MTN-induced vacuolar degeneration and necrosis in the rat liver (Fig. 3A). These changes are entirely consistent with the changes in various biochemical parameters that were also observed such as oxidative stress markers and antioxidant enzyme activities (Figs. 1 and 2). Such liver damage may arise from the toxic effects of MTN, which disturbs the detoxification mechanisms of the liver. In addition, it is possible that MTN, like several other insecticides, adversely affects the cytochrome P450 system or the mitochondrial membrane transport system of hepatocytes (Gokcimen *et al*., 2007). Our study determined that, in concurrent treatment with MTN, the supplementation with α-tocopherol/Se prevents this damage, suggesting that the toxic effect of MTN on hepatocytes is mediated mainly by ROS generation. ROS are thought to be neutralized with α-tocopherol/Se, since damages are not observed in MTN-treated individuals that previously received α-tocopherol/Se.

ROS can be detoxified by an elaborated battery of enzymatic defence system, comprising SOD, CAT, and selenium-dependent GPx, or non-enzymatic systems by the scavenging action of GSH, while organic peroxides can be detoxified by the activity of glutathione S-transferase (GST) (Halliwell and Gutteridge, 1999). The modulation of these enzymes and GSH levels plays a primary role in the balance of the redox status through the reduction of ROS and peroxides produced in the organism, as well as in the detoxification of xenobiotics (Ramiro-Puig *et al*., 2007; Ramos, 2008). OPIs have previously been reported to cause a significant reduction in the activity of key antioxidant enzymes (Pierrefiche and Laborit, 1995). These include SOD, an enzymatic defensive system which permits the dismutation of the superoxide ion ($O_2^{\cdot-}$) into H_2O_2, the accumulation of which is avoided by the catalase/glutathion peroxidase (CAT/GPx) system by transforming it into water and molecular oxygen or oxidized glutathione (GSSH), respectively (Pierrefiche and Laborit, 1995). The most important metabolic roles of selenium in mammalian cell is due to its function in the active site of many antioxidant enzymes, such as GPx (Halliwell and Gutteridge, 1999). The GPx enzyme was the first established selenoenzyme that can prevent oxidative damage of cell membranes. Moreover, GPx does not only protect cells against damages by ROS, but also protects membrane lipids against such oxidation generated by peroxides and permits regeneration of membrane lipid molecules through reacylation (Halliwell and Gutteridge, 1999). Thus, GPx may prevent the harmful

effects of free radicals and may also reduce the formation of the reactive metabolites induced by MTN. When these systems fail or get surpassed, there is an overproduction of $O_2^{\cdot-}$ and H_2O_2 which, when not completely detoxified, give rise to the highly toxic hydroxyl radical ($HO^{\cdot}$). It is known that xenobiotics can induce mitochondria superoxide radical production (Peixoto *et al*., 2004) and if additionally SOD is inhibited the amount of $O_2^{\cdot-}$ formed in cell can reach dangerous levels. Superoxide radical is a potent inhibitor of CAT (Ashakumari and Vijayammal, 1996). Therefore, the observed depletion of antioxidant enzyme activity in this study (Fig. 2) could be caused by a direct effect on the enzyme by MTN-induced ROS generation, depletion of the enzyme substrates and down-regulation of transcription and translation processes.

Many studies have reported an association between human cancer and exposure to the OPIs such as malathion and parathion (Flack *et al*., 1992; National Cancer Institute, 1978). In this context, we have investigated the impact of MTN treatment on the expression of selected apoptosis-related genes, namely: capsase-3 (*CASP3*), caspase-9 (*CASP9*) and tumor protein 53 (*Tp53*). In this study, our results revealed that subchronic exposure of rats to MTN for 45 days is associated with a significant dose-dependent up-regulation of the expression of apoptosis related genes: i.e., *Tp53* (Fig. 4A), *CASP3* (Fig. 4B) and *CASP9* (Fig. 4C), compared with the control. Our findings are supported by recent *in vitro* studies on human breast epithelia cells which have revealed that the expression of cancer-related genes, i.e. *Tp53* inducible protein 3 and *TP53* (Li-Fraumeni syndrome) is up-regulated by malathion and parathion treatments (Calaf and Roy, 2008). Moreover, the *c-Ha-ras* oncogene and several other cell cycle related genes have been shown to be induced by the treatment of malathion alone and with the combination of estrogen and either malathion or parathion (Calaf and Roy, 2008). Furthermore, OPIs mentioned have been shown to cause alterations in cell proliferation and induction of cellular transformation (Calaf and Roy, 2008). Recently, reports have shown that the *CASP3* gene was up-regulated when rat cerebellar granule neurons (CGNs) were exposed to neurotoxic chemicals. In this context, strong correlations have been reported between the transcriptional activity of *CASP3* and the activity of some other genes related to apoptosis, cell-cycle and ROS detoxification. Moreover, pro-apoptotic xenobiotics have been found to CGNs to significantly modulate *CASP3* gene expression (Folch *et al*., 2010).

In neuronal cells, a highly complex apoptotic death process is activated by various endogenous factors and signals from the cell environment. For example, the exposure of neuronal cells to toxic xenobiotics activates key hallmark events such as mitochondrial alteration and DNA damage, which trigger the apoptotic process. Apoptosis has been related to mitochondrial or DNA damage, or cell-cycle activation. The classical apoptotic death process involves the generation of a permeability transition pore complex (PTPC) that permits the release of such proteins as cytochrome c, apoptosis inducing factor (AIF), endonuclease G, and the second mitochondria-derived activator of caspase, Smac (DIABLO) (Xiang *et al*., 1996; Uberti *et al*., 1998; Sakhi *et al*., 1996). The release of cytochrome-c into the cytoplasm induces the formation of the apopotosome complex (Xiang *et al*., 1996; Uberti *et al*.,1998; Sakhi *et al*., 1996; Smith *et al*., 2006), which participates in the activation of the initiator procaspase-9. Subsequently, the caspase cascade is successively amplified *via* the activation of executioner caspases (e.g. caspase-3, caspase-6, and caspase-7) (Kruman *et al*., 2004). Moreover, our findings clearly indicated that the supplementation of α-tocopherol/Se significantly modulated the transcriptional activity of key apoptosis-related genes (Fig. 4). This might be carried out through the potentially efficient antioxidant role of α-tocopherol/Se and ROS neutralizing capacity, whereby preventing damage to DNA and cellular components leading to the attenuation of apoptosis-related pathway (Sies and Murphy, 1991; Packer, 1991; Thomson, 2004; Akhtar *et al*., 2009).

In the present study, the levels of key antioxidant enzymes and compounds were normalised to control in rats supplemented with α-tocopherol/Se as compared to rats given MTN alone. A protective effect of the supplementation with α-tocopherol/Se on histopathological studies was also evident in rat hepatocytes. Moreover, the supplementation of MTN-treated rats with α-tocopherol/Se was characterized by a modulatory impact on the transcriptional activity of key apoptosis-related gene, indicative of an interfering protective role in MTN-mediated poisoning signaling transduction process. Taken together, it may be suggested that poisoning and oxidative stress characteristic of subchronic exposure to MTN may be partially ameliorated by supplementation with α-tocopherol/Se.

ACKNOWLEDGMENT

The authors extend their appreciation to the Deanship of Scientific Research at King Saud University for funding the work through the research group project No RGP-VPP-130.

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