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RESEARCH ARTICLE

Effects of phoxim-induced hepatotoxicity on SD rats and the protection of vitamin E

Jing Zhang[1] · **Wentao Song**[1] · **Yuecheng Sun**[1] · **Anshan Shan**[1]

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Abstract Currently, public pay more attention to the adverse effect of organophosphate pesticides on human and animal health and on the environment in developing nations. Vitamin E may protect the hepatocyte and increase the function of liver. The study was to investigate the effects of phoxim-induced hepatotoxicity on Sprague Dawley (SD) rats and the protection of vitamin E. SD rats received by gavage 180 mg kg^{-1} (per body weight) of phoxim, 200 mg kg^{-1} (per body weight) of vitamin E, and phoxim + vitamin E. The results showed that exposure to phoxim elevated liver coefficient; glutamyl transpeptidase (GGT), aspartate aminotransferase, alkaline phosphatase, total bilirubin, total bile acid, and alanine aminotransferase in the serum; ROS in the liver; and the expression of p53, Bax, CYP2E1, ROS, caspase-9, caspase-8, and caspase-3, while phoxim caused a reduction of total protein, albumin, and cholinesterase in the serum; acetylcholinesterase, total antioxidant capacity, glutathione peroxidase, and glutathione in the liver; and the expression of Bcl-2. Vitamin E modified the phoxim-induced hepatotoxicity by reducing the GGT in the serum, malondialdehyde in the liver, and the expression of CYP2E1 significantly. There were no significant changes of globulin in the serum, the activity of catalase in the liver, as well as expression levels of Fas and Bad in the liver. Overall, subacute exposure to phoxim induced hepatic injury, oxidative stress damage, and cell apoptosis. Vitamin E modified phoxim-induced hepatotoxicity slightly. And, vitamin E minimized oxidative stress damage and ultrastructural changes in rat hepatocytes notably.

Keywords Organophosphate pesticides · Phoxim · Vitamin E · Oxidative stress · Hepatotoxicity · Antioxidant enzymes

Introduction

Organophosphate pesticides (OPPs) are widely used in agriculture, domestic care, stored products, and veterinary care (Celik et al. 2009). The widespread application of OPPs has resulted in a range of ecotoxicological problems, and it ultimately affects mammalian health (Gavelle et al. 2016; Jin et al. 2017). Acute exposure to OPPs mainly inhibits acetylcholinesterase (AChE) activity and even causes death. Exposure to small amounts of OPPs not only inhibits AChE activity (Ogutcu et al. 2008) but also has cumulative effects on hepatotoxicity (Androutsopoulos et al. 2013; Lasram et al. 2014; Elahi et al. 2017). Phoxim is a typical OPP. As a practical application, farmers apply excessive amounts of phoxim to control pests frequently. It is easy to detect phoxim residue in water, soil, and air samples. Moreover, phoxim is used to control the mite population in chickens (Meyer-Kühling et al.

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✉ Anshan Shan
asshan@neau.edu.cn

Jing Zhang
825984292@qq.com

Wentao Song
784874845@qq.com

Yuecheng Sun
371565119@qq.com

[1] Institute of Animal Nutrition, Northeast Agricultural University, Harbin 150030, People's Republic of China

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2007). Recent investigations of phoxim mainly focused on AChE activity, nutrient metabolism, and certain gene expression levels in silkworm (Gu et al. 2015; Li et al. 2016; Wang et al. 2016), while paying less attention to the adverse effects of phoxim at low concentration on mammals.

Vitamin E (α-tocopherols), an essential nutritive element, has potential effects on the mechanisms of health and disease in humans (Catalgol and Ozer 2012). It is thought to be a major lipid-soluble antioxidant and may affect toxicity (Uzunhisarcikli and Kalender 2011; Spodniewska et al. 2015; Mohamed et al. 2017). Vitamin E has a number of biological activities, including attenuating the toxic effects of reactive oxygen species (ROS) in biological systems, scavenging oxygen free radicals effectively (Verma et al. 2007), modifying oxidative stress damage (Hamza et al. 2017), as well as protecting cellular membranes and lipoproteins from peroxidation (Traber and Atkinson 2007; Jia et al. 2017). It is well known that vitamin E is a chain-breaking antioxidant in the reaction of its hydroxyl group with a peroxyl radical (ROO·) (Van Haaften et al. 2003). It allows free radicals to abstract a hydrogen atom from the antioxidant molecule, thus breaking the chain of free radical reactions. And, the resulting antioxidant radical become a relatively unreactive species (Jia et al. 2017).

In general, the liver is the primary organ for bioaccumulation and extensively studied in regard to the toxic effects of xenobiotics. Moreover, oxidative stress effects reflected more efficiently and effectively in the liver (Jin et al. 2010). Ogutcu et al. (2008) believed that OPPs caused an increase in the absolute and relative liver weights were related to enzyme induction of cytochrome P450 (CYP). CYP acts as a terminal oxidase in the mixed-function oxidase system, which metabolizes various endogenous and exogenous substances (e.g., fatty acids, pesticide, and carcinogens) (Fu et al. 2013). It plays an important role in the activation of carcinogens and oxidative detoxification. The liver expresses many CYP isoforms, including CYP2E1 that metabolizes and activates many toxicologically (Jaeschke et al. 2002).

The wide range of applications of phoxim leads to adverse impacts on the health of mammals. Currently, the mechanism and pathway of small amounts of phoxim-induced hepatotoxicity in rats are not clear. Also, there were no studies on whether vitamin E attenuates phoxim-induced hepatotoxicity.

Materials and methods

Chemicals

The phoxim (> 90% pure) was purchased from the Hubei Dixin Chemical Manufacturing Co., Ltd. The substance was stable for at least 1 year. Vitamin E (> 50% pure, DL-α–tocopherol acetate) was supplied by the Vitamin Factory of Zhejiang Medicine Co., Ltd.

Animal

Experimental design

Five-week-old Sprague Dawley (SD) rats of similar weight (140 ± 10 g) were randomly assigned into four treatments of 24 rats per treatment. The phoxim group (phoxim, 180 mg kg^{-1} per body weight) and vitamin E group (vitamin E, 200 mg kg^{-1} per body weight) were administered orally through gavage once a day. Phoxim and vitamin E were dissolved in soybean oil immediately before use. The control group was given the same soybean oil. Vitamin E was administered orally through gavage to the phoxim + vitamin E group 15 min before oral administration of phoxim in soybean oil through gavage. The study started during the 6th week and was completed 4 weeks later.

Animals and treatments

The rats were acclimated for a period of 1 week and had free access to food and water. They were maintained on a 12/12-h light/dark cycle at 25 ± 2 °C in plastic rat cages. The relative humidity was maintained at 47–55%. SD rats were weighed daily, and the requirement of phoxim and vitamin E was calculated. This study was performed in strict accordance with the recommendations of the National Research Council Guide (1996), and all of the animal experimental procedures were approved by the Ethical and Animal Welfare Committee of Heilongjiang Province, China (2008).

Data and sample collection

The animals were sacrificed after 10 weeks. For blood collection, the blood was centrifuged at 3000×*g* for 10 min at 4 °C. Serum layers were removed and placed in sterile microcentrifuge tubes and stored at − 20 °C until assayed. The liver was quickly removed and weighed by electronic balance, and then fragments of the right organs were frozen in liquid nitrogen, stored at − 80 °C, and analyzed for gene expression by RNA extraction followed by quantitative reverse transcription PCR.

Liver coefficient

Body weight and liver weight were measured immediately after sacrifice. The coefficient of the liver was calculated as the ratio of liver weight to body weight:

Liver coefficient (%) = mean of weight of liver for each group / body weight × 100%.

Biochemical parameters

The biochemical parameters were determined with the automated biochemical analyzer (SYNCHRON CX14 PRO model, Beckman Coulter, Inc., USA) using commercial diagnostics kits (Biosino Bio-Technology and Science, Inc., Beijing, China). Parameters were determined for the following biochemical characteristics of serum: aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), total protein (TP), albumin (ALB), globulin (GLB), cholinesterase (ChE), total bilirubin (TBIL), glutamyl transpeptidase (GGT), and total bile acid (TBA).

AChE and ROS

Enzyme activity of AChE in the liver was measured with an ultraviolet spectrophotometer (UV-2410PC model, Shimadzu, Japan) using commercial diagnostics kits (Nanjing Jiancheng Biotechnology Co., Ltd., Jiangsu, China). ROS in the liver was measured with ELISA (JinMa Biological Technology Co., Ltd., Shanghai, China), as well as mRNA expression in the liver (RT-PCR). Nucleotide sequences of primers for RT-PCR, ROS (NM_012874; F: aaaggctgcgtctacttgga; R: cttgactacccgaggactgg).

Antioxidative parameters

The antioxidative parameters were determined with an ultraviolet spectrophotometer (UV-2410PC model, Shimadzu, Japan) using commercial diagnostics kits (Nanjing Jiancheng Biotechnology Co., Ltd., Jiangsu, China). The parameters evaluated in the liver were malondialdehyde (MDA), glutathione (GSH), catalase (CAT), total antioxidant capacity (T-AOC), and glutathione peroxidase (GSH-Px).

Transmission electron microscopy studies

Slices of the liver were double fixed in 2.5% glutaraldehyde and 1% osmium tetroxide, avoiding light at 4 °C. The liver samples were washed four times (15 min each time) with the sodium phosphate buffer (0.1 M, pH 7.2) at 4 °C and postfixed in 1% osmium tetroxide for 1 h at 4 °C. Then, the samples were washed four times (15 min per time) with the sodium phosphate buffer (0.1 M, pH 7.2) and dehydrated in graded ethanol (50, 70, 90, and 100%). The liver tissues were stained with uranium acetate and lead citrate and made into ultrathin sections. The sections were observed under a transmission electron microscope. All the changes were marked.

Quantitative real-time PCR

The total RNA from the liver tissues were isolated using a reagent box (E.Z.N.A.® Total RNA Kit; Omega Bio-Tek, Inc., USA) and according to the reagent instructions. The expression of enzymes and apoptosis genes, including GAPDH (NM_017008.4; F: gagacagccgcatcttcttg, R: tgactgtgccgttgaacttg), CYP2E1 (NM_031543.1; F: tggggaaacagggtaatgag; R: caatcagaaatgtggggtca), Bcl-2 (FQ230631.1; F: ctggtggacaacatcgctctg; R:ggtctgctgacctcacttgtg), caspase-3 (NM012922.2; F: ggagcagttttgtgtgtgtgat; R: atgatgaagagtttcggctttc), caspase-8 (NM_022277.1; F: aggggatgttggaggaagac; R: ctctgggctgctttttagga), caspase-9 (NM031632.1; F: aggctctctggcttcattctt; R: ttctgctcctttgatttgagtc), p53 (NM_030989.3; F: ggacgacaggcagacttttc; R: cagcgtgatgatggtaagga), Bad (NM_022698.1; F: gagcatcgttcagcagca; R: ccatcccttcatcttcctca), Bax (NM017059.2; F: ggatgattgctgatgtggatac; R: ccagttgaagttgccgtctg), and Fas (NM_139194.2; F: cattttgctgtcaaccgtgt; R: agaatagtgtttcctgtccgtgt) were determined through quantitative real-time PCR using an ABI PRISM 7500 SDS thermal cycler apparatus (Applied Biosystems, Foster City, CA, USA).

Statistical analysis

All data are subjected to one-way analysis of variance (ANOVA) by using the least significant difference (LSD) procedure in SPSS (version 20; SPSS Inc., Chicago, IL, USA). Duncan's multiple range test is applied to separate means. All the data are presented as mean ± standard error of the mean (mean ± SEM). Values with different lowercase letter superscripts mean a noble difference ($P < 0.05$), while those with different capital letters show a significant difference ($P < 0.01$).

Results

Liver coefficient

Data describing effects of phoxim-induced hepatotoxicity on the liver coefficient of SD rats and the protection of vitamin E are shown in Table 1. Compared with the control group, liver coefficient significantly increased in the phoxim group and the phoxim + vitamin E group ($P < 0.01$). The liver coefficient of the phoxim + vitamin E group was insignificantly lower than that of the phoxim group ($P > 0.05$).

Table 1 Effects of phoxim-induced hepatotoxicity on the liver coefficient of SD rats and the protection of vitamin E

Items	Control	Vitamin E	Phoxim	Phoxim + vitamin E
Liver coefficient (%)	3.30 ± 0.04^{B}	3.18 ± 0.04^{B}	4.20 ± 0.14^{A}	3.94 ± 0.06^{A}

Values in the table are given as mean±SEM. In the same row, values with different capital letters mean significant difference ($P<0.01$)

Biochemical parameters

Effects of phoxim-induced hepatotoxicity on the biochemical parameters of SD rats and the protection of vitamin E are summarized in Table 2. TP and ALB in the phoxim group were reduced significantly ($P < 0.01$), when compared with the control group. TP and ALB in the treatment of the phoxim + vitamin E group were higher than those of the phoxim group. No statistically significant changes were observed when TP and ALB of the control group was compared with those of the phoxim + vitamin E group. Compared with the control group, ChE was decreased significantly in the phoxim group and the phoxim + vitamin E group ($P < 0.01$). ChE in the phoxim + vitamin E group was lower than that in the phoxim group ($P > 0.05$). The increase of GGT was statistically significant in the phoxim group compared with the other group ($P < 0.01$). Compared with the phoxim group, GGT of the phoxim + vitamin E group decreased notably ($P < 0.01$). Furthermore, the increase in AST, ALP, TBIL, TBA, and ALT activities was statistically significant in the phoxim group compared with that in the control group ($P < 0.01$, $P < 0.05$). Moreover, compared with the phoxim group, the phoxim + vitamin E group showed a downward trend, although this was not significant. There were no significant changes of AST, ALP, TBIL, TBA, and ALT in the phoxim + vitamin E group compared with the control group ($P > 0.05$). Compared with the vitamin E group, GLB in the serum increased significantly in the phoxim group ($P < 0.05$). There were no statistically significant changes observed when the control group was compared with the phoxim group and phoxim + vitamin E group ($P > 0.05$).

AChE and ROS

Compared with the control group, AChE in the liver of the phoxim group and the phoxim + vitamin E group was found to be primarily lower ($P < 0.01$) (Fig. 1a, respectively), while phoxim elevated the ROS in the liver significantly ($P < 0.01$) compared with the control group (Fig. 1b).

Antioxidative parameters

T-AOC, GSH-Px, and GSH were noticeably decreased ($P < 0.01$; $P < 0.05$) in the phoxim group. There were no significant changes between the control group and the phoxim + vitamin E group (Fig. 2a–c, respectively). MDA in the phoxim group increased numerically ($P < 0.01$) compared with the control group. Compared with the phoxim group, MDA decreased significantly ($P < 0.01$) in the phoxim + vitamin E group (Fig. 2d). There was an upward trend of CAT in the phoxim group, although it was not significant ($P > 0.05$) (Fig. 2e).

Transmission electron microscopy studies

Transmission electron micrograph of hepatocytes did not show any pathological changes in the control group and in the vitamin E group. The hepatocytes of rats in the phoxim group displayed dilatation of the endoplasmic reticulum,

Table 2 Effects of phoxim-induced hepatotoxicity on the biochemical parameters of SD rats and the protection of vitamin E

Items	Control	Vitamin E	Phoxim	Phoxim + vitamin E
TP (g/L)	75.79 ± 0.69^{A}	73.69 ± 0.67^{AB}	71.31 ± 0.99^{B}	73.08 ± 1.09^{AB}
ChE (IU/L)	152.29 ± 14.82^{A}	161.33 ± 3.82^{A}	97.75 ± 0.85^{B}	101.5 ± 1.50^{B}
ALB (g/L)	39.78 ± 0.18^{A}	38.10 ± 0.42^{AB}	37.27 ± 0.59^{B}	37.76 ± 0.69^{AB}
GGT (IU/L)	1.37 ± 0.03^{B}	1.44 ± 0.12^{B}	2.40 ± 0.14^{A}	1.78 ± 0.10^{B}
AST (IU/L)	82.46 ± 2.12^{B}	84.33 ± 1.23^{B}	90.50 ± 0.92^{A}	86.42 ± 0.38^{AB}
ALP (IU/L)	150.73 ± 6.42^{B}	156.69 ± 12.41^{AB}	218.09 ± 25.30^{A}	185.73 ± 8.83^{AB}
TBIL (μmol/L)	0.69 ± 0.06^{B}	0.74 ± 0.05^{AB}	1.04 ± 0.10^{A}	0.76 ± 0.06^{AB}
TBA (μmol/L)	11.50 ± 0.78^{b}	12.95 ± 0.27^{ab}	14.86 ± 1.32^{a}	13.69 ± 1.26^{ab}
ALT (IU/L)	28.78 ± 1.42^{b}	28.93 ± 1.31^{b}	33.40 ± 0.77^{a}	31.22 ± 1.14^{ab}
GLB (g/L)	34.70 ± 0.31^{ab}	34.05 ± 0.54^{b}	35.80 ± 0.62^{a}	35.17 ± 0.46^{ab}

Values in the table are given as mean ± SEM. In the same row, values with different lowercase letter superscripts mean noble difference ($P < 0.05$), while those with different capital letters mean significant difference ($P < 0.01$)

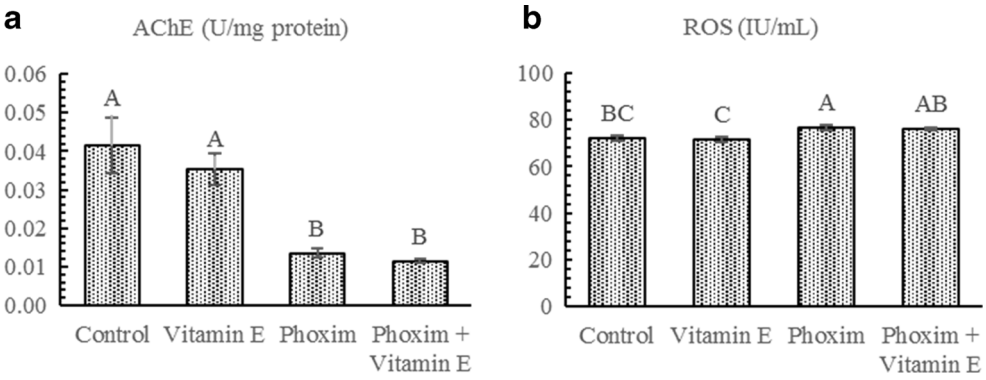

Fig. 1 Effects of phoxim-induced hepatotoxicity on **a** AChE and **b** ROS in the liver of SD rats and the protection of vitamin E

pyknotic nucleus, swelling of mitochondria, and loss of cristae after 4 weeks. It is a nearly complete disintegration of most cellular contents except few numbers of mitochondria, and rough endoplasmic reticulums were observed. Electron micrograph of rat hepatocyte showed weak swelling of mitochondria and weak dilatation of the endoplasmic reticulum in the phoxim + vitamin E group (Fig. 3a–d, respectively).

Quantitative real-time PCR

Phoxim elevated the expression level of p53 and Bax significantly ($P < 0.01$) in the liver of SD rats (Fig. 4a, b, respectively). No distinct changes were observed between the control group and the phoxim + vitamin E group. The expression levels of CYP2E1, ROS, caspase-9, caspase-8, and caspase-3 in the liver of SD rats increased significantly ($P < 0.05$) in the phoxim group compared with the control group (Fig. 4c–g, respectively), while Bcl-2 in the phoxim group was firmly lower ($P < 0.05$) than that in the control group (Fig. 4h). No statistically significant changes of those genes were observed when the control group was compared with the phoxim + vitamin E group (Fig. 4c–h, respectively). Compared with the control group, the downward of Fas and uptrend of Bad were exhibited in the phoxim group ($P > 0.05$) (Fig. 4i, j, respectively).

Discussion

Liver coefficient

OPPs cause serious adverse impacts in rats (Wu et al. 2007; Kalender et al. 2010). Vitamin E, a potential antioxidant, may modify OPP-induced toxicity (Uzunhisarcikli and Kalender 2011; Spodniewska et al. 2015). Liver, a major site for the metabolism of toxic compounds, plays a central role in the detoxification process (Mansour and Mossa 2010; Noh et al. 2015). Organ coefficient relates not only to enzyme induction of cytochrome P450 but also due to food intake, hormonal imbalance, and toxicity (Dirican and Kalender 2012). In our study, liver coefficient was higher in the phoxim group than in the other groups. It may be due to the phoxim-induced hemorrhage, edema, and even inflammation of the liver. Compared with the phoxim group, a downward trend was shown in the phoxim + vitamin E group. It is consistent with Uzunhisarcikli and Kalender (2011), who reported that OPP elevated the relative liver weights of rats.

Biochemical parameters

Biochemical parameters, including ALP, ALT, AST, and GGT, are specific indicators of hepatic function and damage in clinical findings (Abdeldaim et al. 2013; Dalaklioglu et al. 2013). Any interference in these parameters may cause biochemical impairment and lesions of the tissue. OPPs may damage the liver cells and produce hepatotoxicity, such as increasing the enzymatic activities of ALP, ALT, AST, and GGT (Ogutcu et al. 2008; Celik et al. 2009; Kalender et al. 2010). Kalender et al. (2005a) reported that vitamin E decreased diazinon-induced hepatotoxicity by altering biochemical indices in a short period. In this study, ALT, AST, ALP, and GGT activities in the phoxim group increased significantly, compared with the control group. These were consistent with Uzunhisarcikli's report (Uzunhisarcikli and Kalender 2011). It is mainly due to the fact that these cytoplasmic enzymes were secreted into the blood after the structural integrity of the hepatocytes was damaged (Hadi et al. 2012). Compared with the phoxim group, these parameters in the phoxim + vitamin E group had a downward trend.

TP, ALB, and GLB reflect hepatic synthetic function. ChE activity is not only an important factor of acute organophosphorus pesticide poisoning diagnosis and efficacy evaluation but also a clinical reflection of hepatic function. ALB most often transports or binds the toxic compound (Uzunhisarcikli and Kalender 2011). The reduction of ALB means low ability to synthesize protein in the liver. TP is mainly to examine the metabolism of the liver. Uzunhisarcikli and Kalender (2011) have shown that methyl parathion exhibited significantly lower TP and ALB levels at the end of the fourth and seventh

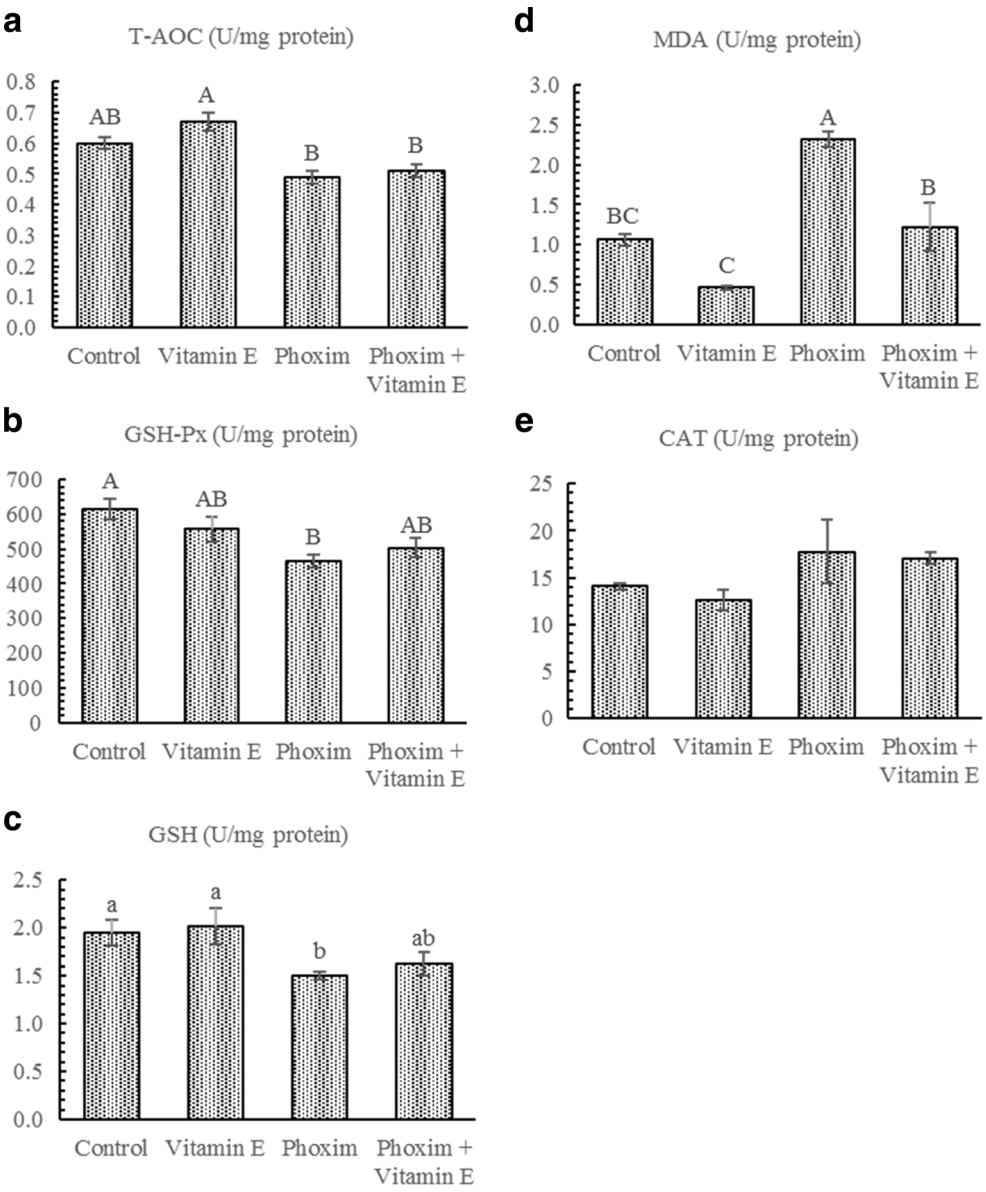

Fig. 2 **a**–**e** Effects of phoxim-induced hepatotoxicity on antioxidative parameters in the liver of SD rats and the protection of vitamin E

weeks. In our study, TP, ALB, and ChE in the phoxim group were reduced markedly, when compared with the control group. Compared with the vitamin E group, the activities of GLB in the serum increased significantly in the phoxim group. There was no statistically significant changes of GLB when the control group was compared with the phoxim group and phoxim + vitamin E group. Phoxim-induced hepatotoxicity not only had adverse impacts on hepatic synthetic function but also inhibited the activities of ChE in serum, while vitamin E could modify it.

Bilirubin is a product of the decomposition and destruction of red blood cells. Toxicant-induced damage of hepatocytes and bile duct cells can cause cholestasis (Jaeschke et al. 2002). In turn, cholestasis leads to intrahepatic accumulation of toxic bile acids and excretion products, which promotes further hepatic injury. Retention of bile constituents within the hepatocyte is related to hepatocyte apoptosis. The failure to secrete bile acids into bile results in liver injury. Bile acids produce hepatocellular apoptosis by stimulating Fas translocation from the cytoplasm to the plasma membrane where self-aggregation occurs to trigger apoptosis (Jaeschke et al. 2002). In this study, TBA and TBIL in the phoxim group increased compared with those of the control group. Adding vitamin E reduced TBA and TBIL activities in the phoxim group.

AChE and ROS

The wide range of applications of OPPs is mainly due to their low toxicity and low persistence in mammals. The primary toxicity in organisms is attributed to the irreversible inhibition of AChE (Costa 2006; Petroianu et al. 2006). AChE is

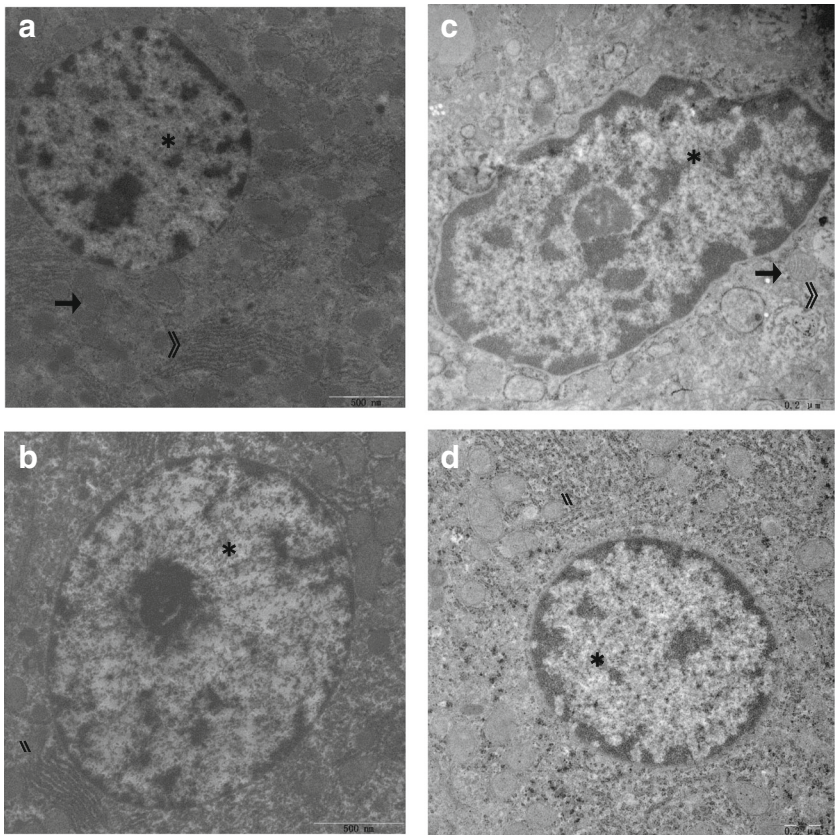

Fig. 3 Effects of phoxim-induced hepatotoxicity on transmission electron microscopy studies in the liver of SD rats and the protection of vitamin E. Transmission electron micrograph of hepatocyte in the control group (**a**), the vitamin E group (**b**), the phoxim group (**c**), and the phoxim + vitamin E group (**d**). There were no pathological changes in the control group and the vitamin E group. Nucleus (asterisk), mitochondria (rightwards arrow), endoplasmic reticulum (right double-angle bracket). Transmission electron micrograph of hepatocyte in the phoxim group showed dilatation of the endoplasmic reticulum (right double-angle bracket), pyknotic nucleus (asterisk), swelling of mitochondria, and cristae (rightwards arrow) that were lost after 4 weeks. It is nearly a complete disintegration of most cellular contents except few numbers of mitochondria, and rough endoplasmic reticulum was observed. Transmission electron micrograph of hepatocyte in the phoxim + vitamin E group showed weak swelling of mitochondria (rightwards arrow) and weak dilatation of the endoplasmic reticulum (right double-angle bracket) after 4 weeks

responsible for the termination of cholinergic impulse by the hydrolysis of acetylcholine (ACh) to choline and acetic acid and plays a vital role in many physiological functions. In our study, compared with the control group, AChE in the liver of the phoxim group was found to be lower mostly, and there were no statistically significant changes in the phoxim + vitamin E group. Vitamin E may release the phoxim-induced inhibition of AChE in the liver. The inhibition occurs because of a nucleophilic reaction between the hydroxyl group of serine in the active site of AChE and the electrophilic phosphorus atom of the organophosphorus compound resulting in the formation of a covalent P–O bond (Vittozzi et al. 2001). Our results indicated that phoxim inhibited the activity of AChE in the liver and vitamin E would lighten the inhibition of AChE.

ROS is produced by cellular respiration in the mitochondria of normal cells. It is necessary to keep the balance between the production and removal of ROS in normal cells. However, the balance shifts towards the former, resulting in oxidative stress. Oxidative stress is implicated as an important pathologic mediator in many disorders, such as liver pathologies (Muriel 2009). Meanwhile, the formation of oxygen free radicals can be a major factor in the toxicity of OPPs (Banerjee et al. 2011). Investigations have shown that OPPs could break down the balance between ROS and antioxidants, triggering the production of ROS and oxidative tissue damage (Storm et al. 2000; Halliwell 2006). It is well known that vitamins prevent the accumulation of ROS in organisms by improving the defense of other antioxidants (Spodniewska et al. 2015). Increased generation of ROS and enhanced lipid peroxidation are considered responsible for the toxicity of a wide range of OPPs (Kalender et al. 2007). Vitamin E neutralizes lipid peroxidation and unsaturated membrane lipids because of its oxygen scavenging effect (Kalender et al. 2010; Dalaklioglu et al. 2013). Our research found that the phoxim elevated the level of ROS in the liver, as well as the expression of ROS in the liver significantly

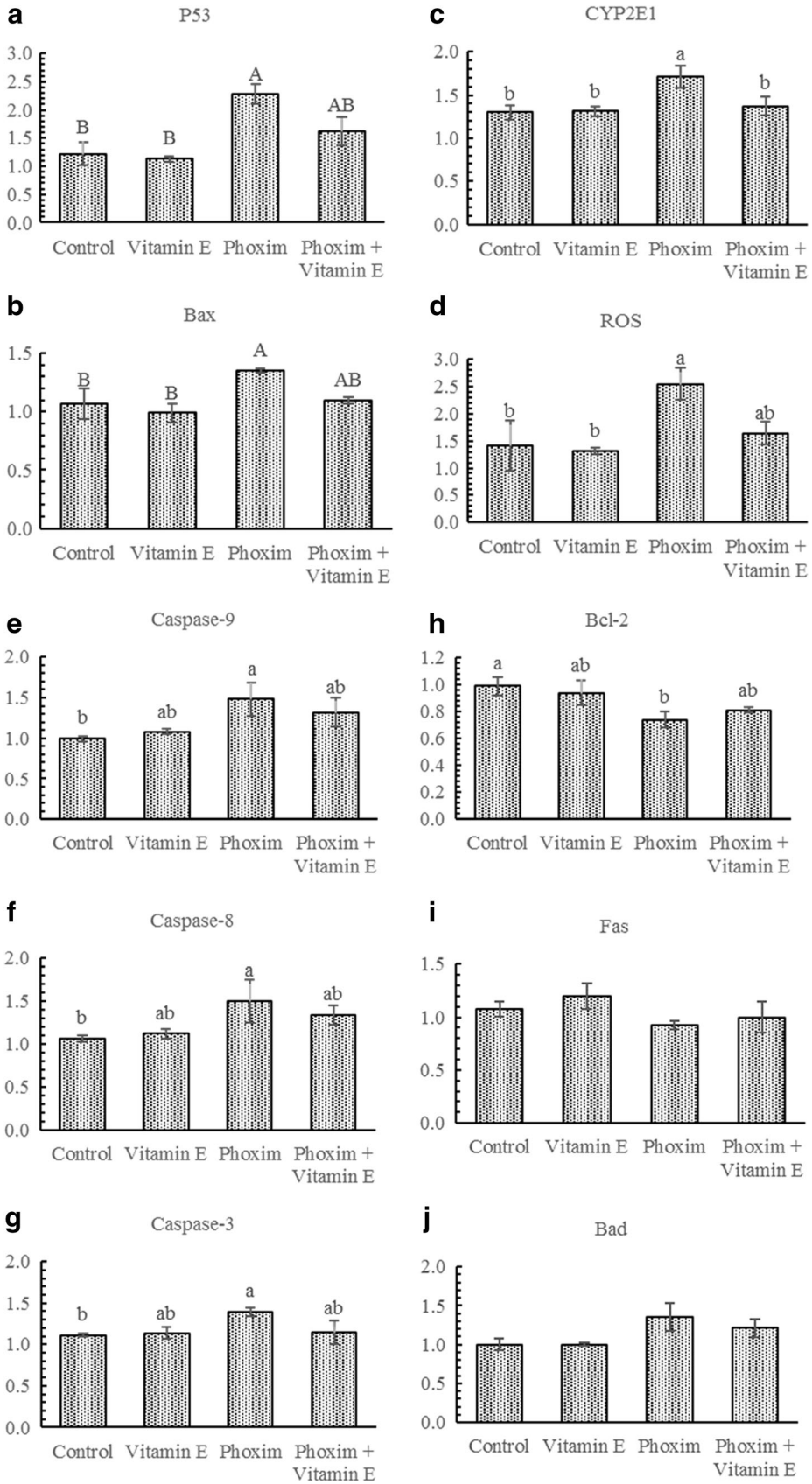

Fig. 4 **a**–**j** Effects of phoxim-induced hepatotoxicity on gene expression levels in the liver of SD rats and the protection of vitamin E

compared with the control group. Vitamin E reduced phoxim-induced ROS in the liver.

Antioxidative parameters

OPPs produce free radicals and cause an increase of lipid peroxidation level in the biological system (Ogutcu et al. 2008). It is necessary to maintain the balance between the production of ROS and antioxidants. When ROS generation exceeds the antioxidant capacity of cells, oxidative stress occurs (Masella et al. 2005). Oxidative stress is known to play a crucial role in liver injury (Videla 2009) and is another mechanism for toxicity of pesticides. It results in cell death (necrosis and apoptosis), and changes in metabolic and vital functions of cells (Soltaninejad and Abdollahi 2009). Recent studies presented that oxidant-antioxidant systems could be affected by OPP toxicity (Cemek et al. 2010a, 2010b). When oxidative stress occurs, the critical cellular antioxidant and detoxifying agents, such as GSH and GSH-Px, are depleted, while MDA, a parameter to measure lipid peroxidation, is accumulated (Videla 2009). GSH-Px catalyzes the reduction of hydrogen peroxide (OH·) and fights against lipid peroxidation (Yang et al. 2011). GSH is known to protect the cellular system against the toxic effects of lipid peroxidation. It is highly important in maintaining cellular redox status. The depletion of GSH is considered as a marker of oxidative stress (Mehany et al. 2013). If GSH and GSH-Px are not sufficient to scavenge ROS products, CAT plays the scavenging role. CAT is the common enzyme in liver and kidney tissues, which increases in the acute phase (Armagan et al. 2015). It is well known that an increase in hepatic MDA levels is an indicator of tissue damage. Han et al. (2017) showed that phoxim induced CAT activity and decreased GSH-Px activity at lower concentrations. In this study, we found that phoxim triggered the oxidative stress of the liver. Compared with other groups, T-AOC, GSH, and GSH-Px were noticeably decreased, while MDA was increased mostly in the phoxim group. However, the increased MDA content might have resulted from an increase of ROS (Li et al. 2015).

Certain antioxidative vitamins can inhibit the generation of free radicals and reduce intracellular oxidative stress. In human, antioxidant therapy has been proposed and investigated for management of pesticide poisoning (Fakhri-Bafghi et al. 2016; Mostafalou and Abdollahi 2017). Vitamin E has received a wide attention due to it neutralizes lipid peroxidation and the toxic effects of ROS. It has hepatoprotective effects on animals. Vitamin E attenuates OPP-induced oxidative stress by reducing MDA levels, restoring the levels of GSH and CAT. Many studies support the use of vitamin E supplementation as a potential therapeutic strategy to fight against oxidative stress (Kalender et al. 2005a, 2005b; Ourique et al. 2016). Södergren's et al. (2001) results indicated that vitamin E suppressed hepatic oxidative injury in rats. Vitamin E reduced the MDA level in heart tissues after diazinon-induced toxicity, but it did not protect completely (Ogutcu et al. 2006). In the present study, adding vitamin E increased phoxim-induced T-AOC, GSH, and GSH-Px levels insignificantly, while it decreased the level of MDA notably in the liver of SD rats. Vitamin E may not completely alleviate phoxim-induced oxidative stress.

Transmission electron microscopy studies

In the normal, transmission electron micrograph of hepatocyte, the nucleus is round and big in the center of hepatocytes. Mitochondria and endoplasmic reticulum are abundant in hepatocytes. The mitochondrion is not only the primary target in OPP-induced cytotoxicity but also the key organelle representing cellular damage (Kalender et al. 2005a). OPP-induced cytotoxicity derives mitochondrial changes, such as swelling of the mitochondria and abnormality of cristae (Joshi et al. 2003). Furthermore, OPPs cause several ultrastructural changes in rat hepatocytes, including dilatation of the endoplasmic reticulum, pyknotic nucleus, and karyolysis of nuclei (Kalender et al. 2005a; Ogutcu et al. 2008; Uzunhisarcikli and Kalender 2011). Dirican and Kalender (2012) found that subacute and subchronic dichlorvos exposure caused mitochondrial vacuolization and swelling. We found phoxim-induced injury in hepatocytes, and the transmission electron microscopy study's findings support the biochemical assays. Phoxim-induced pathological changes include dilatation of the endoplasmic reticulum, pyknotic nucleus, swelling of mitochondria, and the loss of cristae after 4 weeks. Vitamin E recovered phoxim-induced damage mildly in hepatocytes.

Quantitative real-time PCR

CYP2E1 is most efficient in activating environmental contaminants and plays a major role in the activation of oxidative detoxification. CYP2E1 correlates with both lipid peroxidation and the activation of caspase-3 (Jaeschke et al. 2002). When oxidative stress occurs, the level of lipid peroxidation is largely enhanced and leads to an increase in the level of CYP2E1. Caspase-3 is regarded as a major effector caspase in apoptosis (Chen et al. 1997). In this study, we found that phoxim elevated the expression levels of CYP2E1 and caspase-3 significantly in the liver of SD rats. The phoxim + vitamin E group displayed a downward trend of CYP2E1 and caspase-3, when compared with the phoxim group.

Apoptosis plays a dominant role in the pathogenesis of liver injury. An apoptotic effect was seen on exposure to OPPs (Zhang et al. 2011). OPPs may lead to mitochondrial changes and oxidative stress (Uzunhisarcikli and Kalender 2011). However, mitochondrial change causes release of cytochrome c, which activates caspases in the cytosol to cause apoptosis through mitochondrial permeability transition

(MPT). Upon oxidative stress, p53 and Bax are overexpressed at high levels, while GSH is depleted. Bax overexpression can promote MPT and cell death (Erkekoğlu et al. 2011). p53 is a central sensor in response to multiple cellular stress signals in cell apoptosis (Vaseva et al. 2012). Upon activation, p53 leads to cell cycle arrest and promotes DNA repair or induces apoptosis via several pathways (Pflaum et al. 2014). Apoptosis is triggered by activating both the mitochondrion-initiated intrinsic pathway and the death receptor/extrinsic pathway (Zeng et al. 2012). The former, mitochondrion-initiated intrinsic pathway, is associated with Bcl-2 family, caspase-3, and caspase-9. Bcl-2 is a proto-oncogene and blocks cytochrome c release and caspase activation during apoptotic signaling through mitochondria. Caspase-9 activates the executor caspase-3 and eventually leads to apoptosis. The latter, death receptor/extrinsic pathway, is mediated by a variety of death receptor ligands, such as Fas, Fas ligand (FasL), and caspase-8. Fas is the predominant death receptor expressed by hepatocyte. Caspase-8 is an initiator cysteine-aspartate protease in apoptosis. Fas expression is associated with p53 (Elmore 2007; Wilson and Dixit 2009). p53 induces the expression of Fas. When p53 accumulates in the cytosol, it can activate Bax and trigger apoptosis directly. Our study found that phoxim elevated the expression levels of p53, Bax, caspase-9, and caspase-8 significantly in the liver of SD rats, while it decreased the expression level of Bcl-2. It means intrinsic apoptosis pathways were activated by phoxim. Compared with the control group, the downtrend of Fas and uptrend of Bad were exhibited in the phoxim group. Vitamin E may alter the expression of certain genes.

In conclusion, subacute exposure to 180 mg kg^{-1} (per body weight) of phoxim-induced hepatotoxicity mainly triggered hepatic injury, oxidative stress damage, and cell apoptosis via mitochondrion-initiated intrinsic pathway in SD rats. Vitamin E attenuated phoxim-induced hepatotoxicity, mainly on oxidative stress damage and ultrastructural changes in rat hepatocytes.

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Compliance with ethical standards This study was performed in strict accordance with the recommendations of the National Research Council Guide (1996), and all of the animal experimental procedures were approved by the Ethical and Animal Welfare Committee of Heilongjiang Province, China (2008).

Conflict of interest The authors declare that they have no conflict of interest.

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