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Research Article

Dose- and Time-dependent Effects of Permethrin on HepG2 Cells: Cell Survival, Lipid Peroxidation and Antioxidant Defence System [1] [2]

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Abstract

There is very limited knowledge about *in vitro* hepatotoxicity of permethrin concerning dose and duration even though humans and non-targeted beings are exposed. In this study, three different doses of permethrin (1 uM, 10 uM, 100 uM) were administered in three different time periods (24, 48, 72 h) and cell viability (WST-1 and Trypan blue test), lipid peroxidation (high performance lipid chromatography), and antioxidant (SOD-1, SOD-2 and GPx-1) gene expression levels (real time PCR) were evaluated. The LC50 dose of permethrin was calculated as 1111 µM. Significant decrease in cell viability was detected in every time period except at the lowest dose ($P<0.05$). Each permethrin dose caused a significant increase ($P<0.01$) in superoxide dismutase-1 levels (except 1 µM at 48 h). The 10 µM and 100 µM groups' superoxide dismutase-2 levels were higher than the controls at each exposure level though the 1 µM group was significantly lower at 24 and 48 h and higher at 72 h. Interestingly, a non-uniform statistically significant difference for glutathione peroxidase-1 was seen in each exposure duration and doses either as up- or down regulation ($P<0.01$). Generally, malondialdehyde concentrations were significantly increased ($P<0.01$), although at each dose in 72 h a significant decrease in malonaldehyde levels was seen ($P<0.01$). Our results may help in understanding the molecular aspects of high dose permethrin hepatotoxicity. More comprehensive research is required to evaluate long term low dose exposure.

Keywords: *Cytotoxicity, HepG2, Oxidative stress, Permethrin*

Permetrin'in HepG2 Hücrelerine Doza ve Zamana Bağlı Etkileri: Hücre Sağkalımı, Lipid Peroksidasyonu ve Antioksidan Savunma Sistemi

Öz

Permetrinin tüm dünyada yoğun kullanımıyla insanlar ve diğer hedef dışı canlılar maruz kalmasına ragmen doz ve zamana bağlı *in vitro* hepatotoksisitesi ile ilgili sınırlı bilgi bulunmaktadır. Bu çalışmada, HepG2 hücrelerine permetrinin üç farklı dozu (1 uM, 10 uM, 100 uM) üç farklı zaman aralığında (24, 48, 72 saat) uygulanarak hücre canlılığı (WST-1 ve Tripan Mavisi testi ile), lipid peroksidasyon (yüksek performanslı sıvı kromatografisi ile), ve antioksidan (SOD-1, SOD-2 ve GPx-1) gen ekspresyon düzeylerinin (gerçek zamanlı PCR ile) değerlendirilmesi amaçlanmıştır. Permetrinin LC50 dozu 1111 µM olarak hesaplandı. Tüm zamanlarda en düşük doz hariç hücre canlılığında önemli azalma belirlendi ($P<0.05$). Permetrin'in tüm dozları, tüm maruz kalma sürelerinde (48 saatte 1 µM hariç) süperoksit dismutaz-1 seviyelerinde önemli bir artışa neden oldu ($P<0.01$). Tüm maruziyet sürelerinde 10 µM ve 100 µM grublarında süperoksit dismutaz-2 seviyeleri kontrol grubundan daha yüksek olmasına rağmen, 1 µM grubu 24 saat ve 48 saatte anlamlı olarak düşüktü ve 72 saatte daha yüksekti ($P<0.01$). İlginç olarak Glutatyon peroksidaz-1 için, tüm maruziyet grupları ve maruz kalma sürelerinde kontrole göre uniform olmayan up ya da down regülasyon şeklinde istatistiksel olarak anlamlı fark gözlendi ($P<0.01$). Genel olarak, malondialdehit konsantrasyonları önemli ölçüde artmasına karşın ($P<0.01$), tüm permetrin dozlarında 72 saatte malondialdehit seviyelerinde önemli bir düşüş olmuştur ($P<0.01$). Sonuçlarımız yüksek doz permetrin kaynaklı hepatotoksisitede rol oynayan moleküler yönlerin daha iyi anlaşılmasını sağlayabilir. Düşük doz uzun süreli maruziyet için daha detaylı araştırmalara ihtiyaç vardır.

Anahtar sözcükler: *Sitotoksisite, HepG2, Oksidatif stres, Permetrin*

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INTRODUCTION

Permethrin (PER), a synthetic pyrethroid insecticide is commonly used for many purposes, such as agriculture, public health, forestry, veterinary medicine, and vector control programs, due to its high activity as an insecticide and low mammalian toxicity [1,2]. Approximately 2 million pounds of PER is used annually in the fields of agriculture, housing and public health, mostly in non-agricultural areas (70%) [3]. However, this extensive use increases its residual contamination in the environment and potential for human and animal exposure [2]. In addition, a number of studies have confirmed its carcinogenic potential [4,5] as well as generation of oxidative damage [2] in vertebrates and invertebrates.

Permethrin acts on the nervous system of target organisms. It forms damage in function of voltage-gated sodium channels in the neurons, and causes paralyses and eventually death with muscular spasm. Compared to those of insects, mammalian sodium channels are less sensitive to effects of pyrethroids because of rapid recovering [6]. Also, pyrethroids have low hydrolytic activity, and they are easily converted to non-toxic derivatives thorough hydrolytic activity in mammalian species in contrast to insects [7].

The liver is an important organ in the detoxification of xenobiotics, and it is known that PER is a potent inhibitor for cytochrome P4501A, resulting in substantial accumulation of some chemicals associated with fatal toxicities [8,9]. PER also causes some pathological alterations in hepatocytes, reducing the size of the nuclei and causing hydropic degeneration of the hepatocytes [10]. A previous study showed imbalance and damage in the redox system of the liver of rats in adolescence (subchronic) and neonatal (subacute) age following PER treatment [8].

Although the action of pyrethroids on various target cells is different, oxidative stress (OS), reactive oxygen species (ROS) and reactive nitrogen species (RNS) associated with PER may play critical roles in induction of a variety of toxicities in humans and animals, including neurotoxicity [11], immunotoxicity [12], cardiotoxicity [13], hepatotoxicity [8,10,14,15], genotoxicity [16], and cytotoxicity [10,17]. It has been shown that PER induces a significant increase in OS in various types of cells such as erythrocytes, leukocytes, brain cells, heart cells, smooth muscle cells, thymic cells and lymphocytes [2].

HepG2 is a human hepatocellular carcinoma cell line with high proliferation capacity and many differentiated hepatic functions that can metabolically transform initial substances by their endogenously overexpressed oxygenases [18]. HepG2 cell monoculture or co-culture models have been most commonly used in drug metabolism and hepatotoxicity studies for the prediction of pesticide-induced liver injury, as *in vitro* alternative to primary human hepatocytes [18-21].

A literature search revealed there is no report regarding OS associated with PER in the HepG2 cell line. In the present study, investigation of PER induced OS in human hepatoma HepG2 cells for further understanding the mechanisms of PER toxicity was aimed. The effect of different doses of PER on cell viability, lipid peroxidation and antioxidant enzymes were assessed in a time-dependent manner. For this purpose, the measurement of malondialdehyde (MDA) level as a marker of lipid peroxidation was analysed by high performance lipid chromatography (HPLC); and the gene expression of superoxide dismutase-1 (SOD-1), superoxide dismutase-2 (SOD-2) and glutathione peroxidase-1 (GPX-1) were detected by real time PCR.

MATERIAL and METHODS

Cell Culture, WST Assay and Treatment

The human HepG2 hepatocellular carcinoma cell line was provided by American Type Culture Collection (ATCC, HB-8065, Manassas, VA, USA). The cells were grown in Eagle's Minimum Essential Medium (EMEM) (M0275, Sigma, USA) which was supplemented with 10% fetal bovine serum (FBS) (10500064, Gibco), 2 mM L-glutamine (G7513, Sigma USA), 100U/mL penicillin/streptomycin (15140122, Gibco) and 1% non-essential amino acids (M7145, Sigma USA) at 37°C in a humidified incubator with a 5% CO_2 atmosphere.

Firstly, PER (25:75 is a mixture of cis and trans isomers, 3-phenoxybenzyl (IRS,3RS;1RS,3SR)-3-(2,2-dichlorovinyl) -2,2-dimethylcyclopropanecarboxylate, 100% purity, Y0001733, European Pharmacopoeia, Strasbourg, France) was dissolved in dimethyl sulfoxide (DMSO, D2650, Sigma Chemical Co., St. Louis, MO, UK) at a series stock solutions concentration of 1, 10 and 100 mM. The stock solutions were diluted using culture medium in the range of 1-10000 µM. Lethal concentration50 (LC50) of PER was calculated using a colorimetric assay for 96-well plates with 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt (WST-1, 05015944001, Roche Diagnostics, Germany) reagent. For WST-1 assays, HepG2 cells were subcultured in 96-well plates at a seeding density $3x10^4$ cells per well for 24 h. After incubation the medium was changed and the cells were treated with 100 µL different concentrations of PER for 24 h. Additionally each plate contained blanks, controls (no treatment), and vehicle (DMSO, 0.1%) with three replicates each. Then, 10 µL of WST-1 reactant was added to each well and incubated for 30 min. Cell viability was measured at 450 nm in a microplate reader spectrophotometer (Tecan software Magellan, Switzerland).

Cells ($3x10^5$, total cell count of per mL) were treated with three different doses of PER 1 µM, 10 µM and 100 µM (approximately 1/1000, 1/100 and 1/10 of the determined LC50) and at three different time intervals (24, 48 and 72 h). The treatments were performed as three replicates and all the experiments were repeated four times.

HepG2 Cell Viability Assay

The trypan blue exclusion test was used to determine the number of viable cells. Briefly, HepG2 cells were treated with three different doses of PER (1 µM, 10 µM and 100 µM) and at three different time intervals (24, 48 and 72 h) as well as control (no treatment) and DMSO (0.1% vehicle) treatment. Cells were harvested, and then aliquot of 10 µL of the cell's suspension was added with similar volume of 0.4% trypan blue solution (T8154, Sigma-Aldrich, St Louis, MO, USA). Cell viability was analyzed using an automated cell counter (TC-20™, Bio-Rad Laboratories, Hercules, CA, USA).

Lipid Peroxidation Measurements

MDA levels in the cell supernatant were determined with 2,4-dinitrophenylhydrazine (DNPH, D199303) derivative method by HPLC [22]. All analytical grade chemicals were provided by Sigma-Aldrich, St Louis, MO, USA. External standards were prepared in the range of 0.3-10 nmol/mL using 1,1,3,3-tetraethoxypropane (TEP, T9889) in 1% sulphuric acid. Cell supernatant samples (125 µL) were treated with 25 µL 6M aqueous sodium hydroxide and the mixture was incubated at 60°C for 30 min. For precipitation of proteins, 35% perchloric acid was added and centrifuged at 2800 g for 10 min. The supernatant was transferred to the eppendorf tube and then added the DNPH prepared in hydrochloric acid. This mixture was allowed to incubate for 30 min at room temperature in a dark environment. An aliquot of 50 µL of this reaction mixture was injected into the HPLC equipment (Shimadzu, LC-20AT Prominence, Kyoto, Japan) with a photodiode array detector (SPD-M20A, 310 nm). The separation was performed with an ACE 5 C18 column (4.0×125 mm x 5 µm particle size (ACT, Scotland). The mobile phase consisted of 0.2% acetic acid in deionised water (v/v) and acetonitrile/water (62:38, v/v). The flow rate was 0.6 mL/min. The linearity was satisfactory (R^2 = 0.999) and the limit of detection (LOD) and limit of quantification (LOQ) values were 0.003 nmol/mL and 0.01 nmol/mL, respectively. Determination of the protein content was based on the Bradford method [23], using the Bradford reagent (B6916). MDA level was expressed per mg protein.

mRNA Quantification of Antioxidant Enzymes (SOD-1, SOD-2 and GPX-1)

Total RNA was isolated using the RNA Isolation Kit (PureLink RNA mini kit, 12183018A, Ambion), according to the manufacturer's instructions. Purity and concentration of the isolated RNA were detected by measuring absorption at 260-280 nm spectrophotometrically. cDNA synthesis was performed with High-Capacity cDNA Reverse Transcription Kit (4368814, Applied Biosystems), according to the manufacturer's instructions. cDNA samples were stored at -20°C until use. Quantitative RT-PCR was performed in triplicate with the LightCycler 1.5 instrument (Roche Diagnostics, Mannheim, Germany) using LightCycler TagMan Master Mix and Real Time ready single assays primer-probe sets (04735536001, Roche Diagnostics, Mannheim, Germany). The RT-PCR conditions consisted of initial denaturation phase at 95°C for 10 min, followed by 45 cycles at 95°C for 10 sec, 60°C for 30 sec and 72°C for one sec. Analysis and quantification were performed using LightCycler 480 software. Relative quantification was calculated by delta Ct method, and SOD-1, SOD-2 and GPX-1 expressions were normalized with respect to the expression of an endogenous control, beta-actin.

Statistical Analysis

The normality of the data was evaluated with the Kolmogorov-Smirnov Test. For all parameters, comparisons of the doses in each experiment time were made with One-Way Analysis of Variance (ANOVA) followed by Tukey's and Duncan tests. All data were expressed as mean ± standard error (SEM). Differences were considered statistically significant when P values were less than 0.05.

RESULTS

WST-1 Assay

Between the vehicle-treated (DMSO) and control cells, there were no significant differences for cell viability. PER resulted in a significant decrease in cell viability in a concentration-dependent manner compared to the control group *(Fig. 1)*. The LC50 value was calculated as 1111 µM for PER.

Cell Viability

When evaluating the results of the trypan blue test for cell viability, there was a statistically significant reduction in cell viabilities at 10 µM and 100 µM PER doses for all three-exposure times (P<0.05). However, the 1 µM treatment dose did not induce significant cell loss compared to

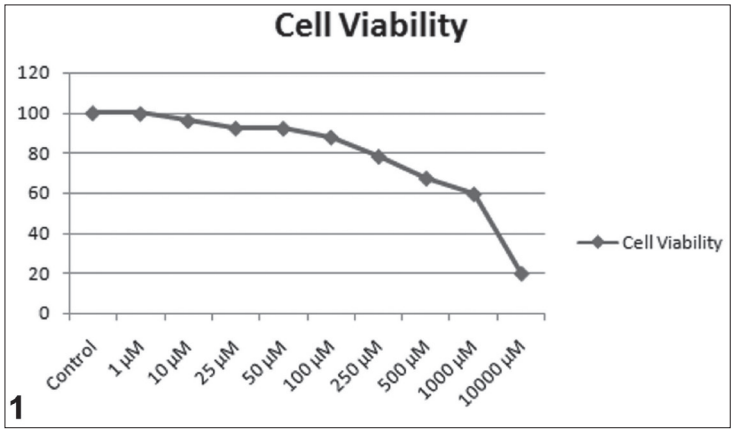

Fig 1. Cytotoxicity of HepG2 cell culture after 24 h exposure time to different concentration of permethrin. Each point represents the average of three independent experiments on cultures of HepG2 cells. Means are plotted

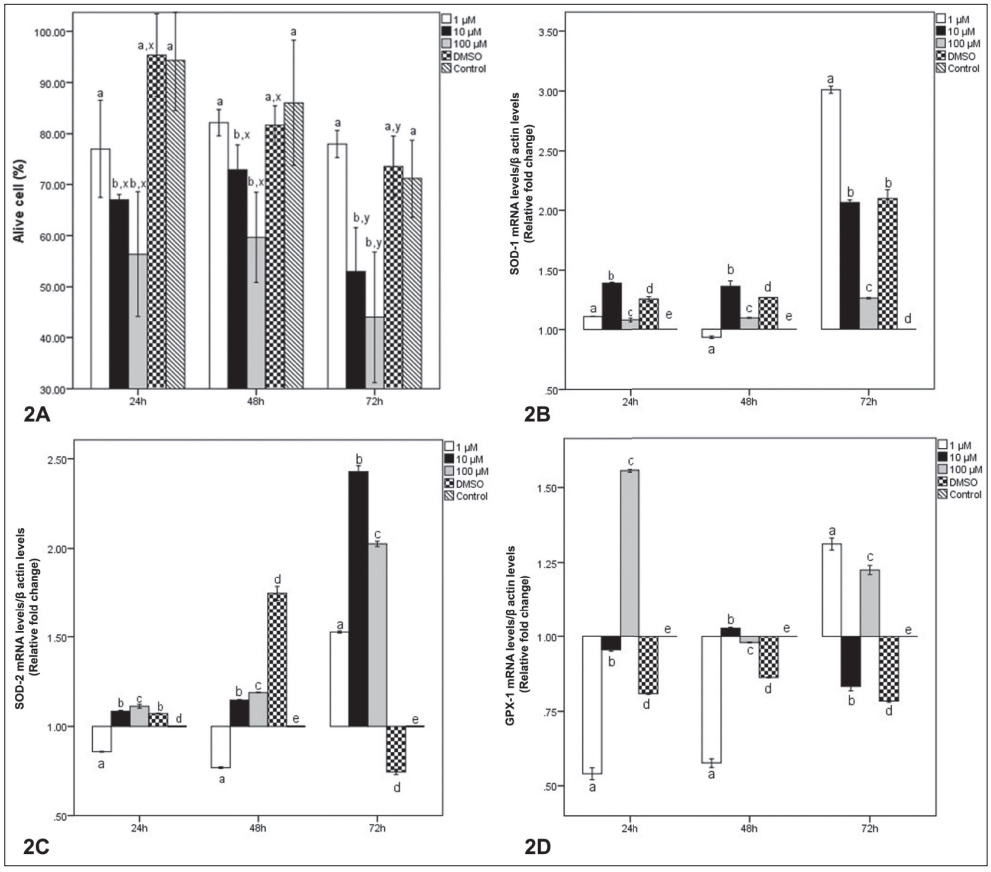

Fig 2. Effects of Permethrin on cell viability and the level of each amplified cDNA was normalized to that of B-actin and represented as the bar graph. A) Cell viability ([a,b] Statistical comparisons of alive cell ratio of groups in each experimental time, [x,y] Time-dependent changes of each group on alive cell ratio, P<0.01) B) SOD-1 mRNA levels C) SOD-2 mRNA levels D) GPX-1 mRNA levels ([a,b,c,d,e] Statistical comparisons of fold changes of mRNA of groups in each experimental time, P<0.01)

Table 1. *MDA (malondialdehyde) levels in PER (permethrin) treated HEPG2 cell line*

Dose	MDA Level (nmol/mg protein)		
	24 h	48 h	72 h
1 µM	0.266±0.015[a]	8.566±0.025[a]	1.253±0.045[a]
10 µM	0.223±0.015[b]	6.466±0.135[b]	0.800±0.040[b]
100 µM	1.853±0.025[c]	5.503±0.075[c]	0.380±0.060[c]
DMSO	0.050±0.01[d]	1.113±0.123[d]	1.413±0.075[d]
Control	0.020±0.01[d]	0.490±0.081[e]	1.516±0.080[e]

Data are expressed as mean ± SEM; [a,b,c,d,e] Means with different superscript in the same column differ significant (P<0.01)

the control and DMSO groups (P>0.05). Time-dependent changes on the cell viability were significantly different in 10 µM, 100 µM and DMSO groups at 72 h (P<0.05) *(Fig. 2-A)*.

Lipid Peroxidation

A significant increase in the MDA concentration of the PER groups was observed at 24 h and 48 h (P<0.01). However, there was a significant decrease in MDA levels at 72 h in all PER doses compared to control and DMSO groups (P<0.01). The alterations in the MDA concentrations were not dose-dependent and the highest level of MDA was detected at the 1 µM dose for all exposure times *(Table 1)*.

mRNA Expression of Antioxidant Enzymes (SOD-1, SOD-2 and GPX-1)

In general, statistically important differences were observed between OS parameters of all treatment groups at all exposure times (P<0.01). However, there was no difference between 10 µM and DMSO groups at certain times (24 h for SOD-2 and 72 h for SOD-1) (P>0.05).

All doses of PER caused a significant increase (up regulation) in SOD-1 levels at all exposure times except 1 µM (down-regulation) at 48 h compared to controls (P<0.01) *(Fig. 2-B)*. SOD-2 levels of the 10 µM and 100 µM groups were upregulated at all exposure times. However, SOD-2 levels of the 1 µM group were significantly downregulated at 24 h and 48 h and upregulated at 72 h compared to those of control groups (P<0.01) *(Fig. 2-C)*. Although, compare to control groups for expression of GPX-1 statistically significant difference (up-or down regulated) were observed in all treatment groups (P<0.01), these expression levels did not have uniform distribution *(Fig. 2-D)*.

In terms of changes in SOD-1, SOD-2 and GPX-1 levels of groups at three exposure times, time-dependent changes (up or down regulation) were observed for all PER groups and the DMSO group. These changes were determined statistically important in all doses (except 1 µM) at all exposure times for SOD-2 and GPX-1 and only at 72 h for SOD1 ($P<0.01$).

DISCUSSION

Permethrin, one of the type I synthetic pyrethroid insecticides, has been widely used in the world due to its high insecticide activities and low toxicity for mammals. However, increasing studies indicate that PER-related toxic effects arise from OS in various toxicity models under many *in vitro* and *in vivo* conditions [2]. In addition, several studies reported that PER and other synthetic pyrethroids have cytotoxic effects on different cell lines in certain doses [10,21,24]. Similarly, PER has dose-dependent cytotoxic effects on HepG2 cells in the present study. The alive cell ratio of 1 µM dose was not different from the DMSO and control groups and any cytotoxicity were not determined at the lowest dose. However, the 10 µM and 100 µM doses caused significant cytotoxicity and this effect was maximally observed at 72 h time period.

Lipid peroxidation, damage to cell membranes, lipoproteins, and other lipid-containing structures triggered by OS and generation of ROS or RNS, is a well-known process associated with various pathological conditions and accelerated aging, leading to molecular cell damage, such as necrosis or apoptosis [25].

Cells in response to membrane lipid peroxidation either stimulate survival or induce cell death processes, and this situation depends on cellular metabolic conditions and repair capacities. In the case of low lipid peroxidation (sub-toxic conditions), cells stimulate survival by providing adaptive stress responses, such as antioxidant defence systems or signalling pathways. In the case of moderate or high lipid peroxidation (toxic conditions), oxidative damage exceeds repair capacity and cell death, such as apoptosis/autophagy/necrosis, is induced. In the present study, adaptive responses were insufficient in 10 µM and 100 µM doses at 72 h, these situations could be explained by low number of alive cells. The peroxidation of lipids usually takes place in three stages: initiation, propagation and termination. In the early stages, lipid radicals and lipid hydroperoxides occur, whereas in later stages, aldehyde products such as MDA are formed [26,27]. In our study, an increase in MDA levels was observed in all doses from 24 h to 48 h, which may indicate that the 24 h and 48 h are the early stage and the late stage of lipid peroxidation, respectively.

Lipid peroxidation yields a wide variety of oxidation products including many different aldehydes, such as MDA, propanal, hexanal, and 4-hydroxynonenal. MDA is one of the final products of polyunsaturated fatty acids, such as omega-3 and omega-6 fatty acids, and is accepted as a convenient lipid peroxidation marker [25,28]. In a rat model of dermal PER exposure, significant increases in plasma MDA concentrations are associated directly with tissue damage [29]. In general, MDA levels of PER groups were determined to be higher than the DMSO and control groups in this study. Increased MDA was not dose-dependent and the highest MDA levels were observed in 100 µM and 1 µM doses at 24 h and 48 h, respectively. In contrast, MDA levels of PER groups were significantly lower than the DMSO and control at groups 72 h. Previous studies showed that PER and analogues (its enantiomers) caused dose-dependent increases of MDA in cell lines [17,20,30]. In our study, increased MDA could be assessed as the lipid peroxidation effect of PER, as well. However, the impact of doses on the MDA level was not observed at 24 h and 48 h. Possible explanations for decreases in the MDA levels at 72 h post-exposure is that it was enzymatically metabolized or it might react on cellular proteins or DNA to form adducts, resulting in damage at the molecular level 72 h after PER exposure [25]. In our study, cell viability results showed that cytotoxicity (via apoptosis and/or autophagy) was observed predominant in time and decreased alive cell ratios could be linked to decreased production of MDA. Also, MDA may bind with some other cellular products, and therefore, it can not be visible after a certain time. In addition, extra stress caused by several changes of the cell medium may cause the increased MDA levels of the DMSO and control groups at 72 h [25-27,30].

Oxidative stress is the imbalance between harmful ROS and the antioxidant defence system. ROS can damage DNA, protein and membrane lipids and cause aging, cancer and various diseases. There is a close link between cellular responses to OS and antioxidant enzyme levels. Commonly used enzymes for evaluation of the OS status are SOD and GPX. Effects of synthetic pyrethroids on OS parameters have been investigated in several papers, and it was reported that single or combined exposure to synthetic pyrethroids resulted in *in vitro* OS [17,20,27,31]. Similar with MDA levels, a general increase in antioxidant enzyme levels was observed in the present study. Also, dependence on the dose was not determined at each exposure time. The obtained findings may be evaluated, as PER caused OS in HepG2 cells, but detailed studies should be performed for investigation of the effects of different doses and time periods. *In vivo* studies also showed that PER dose-dependently induces OS in rat liver, reducing antioxidant enzyme activities including SOD and GPX, and finally leading to the lipid and protein peroxidation thought to be crucial for hepatotoxicity [8,14].

In conclusion, similar results from both the present study and previous *in vivo* studies showed the effects of PER on redox systems, and the HepG2 cell line might be a useful and reliable non-animal alternative for clarifying mechanisms of

PER-related hepatotoxicity. In addition, further investigations are needed to determine the association of OS in HepG2 cells exposed to PER, which leads to lipid peroxidation, with different pathways, including cell death/apoptosis or autophagy, to be crucial in the cell/tissue damage related to pesticide toxicity.

Conflicts of Interest

There are no conflicts to declare

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