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ATTENUATING EFFECTS OF CAFFEIC ACID PHENETHYL ESTER WITH INTRALIPID ON HEPATOTOXICITY OF CHLORPYRIFOS IN THE CASE OF RATS

ŁAGODZĄCE DZIAŁANIE ESTRU FENETYLOWEGO KWASU KAWOWEGO Z INTRALIPIDEM NA HEPATOTOKSYCZNOŚĆ CHLOROPYRIFOSU U SZCZURÓW

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ABSTRACT

Background: Chlorpyrifos (CPF), insecticide widely used in agriculture, may cause poisonings in the case of humans. As a result, there is a large amount of treatment research underway to focus on the possibility of chlorpyrifos induced poisonings. The aim of this study has been to evaluate the effects of caffeic acid phenethyl ester (CAPE) and intralipid (IL) on hepatotoxicity induced by chlorpyrifos in the case of rats. **Material and Methods:** The rats in this study were treated with CPF (10 mg/kg body weight (b.w.), orally), CAPE (10 µmol/kg b.w., intraperitoneally), IL (18.6 ml/kg b.w., orally), CPF+CAPE, CPF+IL, and CPF+CAPE+IL. The plasma total oxidant capacity (TOC), total antioxidant capacity (TAC) were measured and the oxidative stress index (OSI) was calculated. Liver histopathology and immunohistochemical staining were performed. **Results:** Chlorpyrifos statistically significantly decreased the TAC levels in the rats' plasma and increased the apoptosis and the TOC and OSI levels. In the chlorpyrifos induced liver injury, CAPE and CAPE+IL significantly decreased the plasma OSI levels and the apoptosis, and significantly increased the plasma TAC levels. **Conclusions:** This study revealed that CAPE and CAPE+IL attenuate chlorpyrifos induced liver injuries by decreasing oxidative stress and apoptosis. *Med Pr* 2016;67(6):743–749

Key words: apoptosis, oxidative stress, liver, rats, chlorpyrifos, caffeic acid phenethyl ester

STRESZCZENIE

Wstęp: Chloropiryfos (CPF), środek owadobójczy szeroko stosowany w rolnictwie, może powodować zatrucia u ludzi. Z tego powodu prowadzi się wiele badań dotyczących możliwości leczenia zatrucia chloropiryfosem. Celem pracy była ocena wpływu estru fenetylowego kwasu kawowego (caffeic acid phenethyl ester – CAPE) i intralipidu (IL) na uszkodzenia wątroby u szczurów wywołane przez chloropiryfos. **Materiał i metody:** Szczurom podawano CPF (10 mg/kg masy ciała (mc.), doustnie), CAPE (10 µmol/kg mc., dootrzewnowo), IL (18,6 ml/kg mc., doustnie), CPF+CAPE, CPF+IL oraz CPF+CAPE+IL. Zmierzono całkowitą zdolność utleniającą (total oxidant capacity – TOC) i całkowitą zdolność przeciwutleniającą (total antioxidant capacity – TAC) osocza krwi i obliczono wskaźnik stresu oksydacyjnego (oxidative stress index – OSI). U zwierząt wykonano także badanie histopatologiczne i barwienie immunohistochemiczne tkanek wątroby. **Wyniki:** Chloropiryfos istotnie zmniejszał u badanych szczurów TAC osocza, a zwiększał apoptozę, TOC i OSI. Natomiast CAPE i CAPE+IL istotnie zmniejszały OSI osocza i apoptozę, a zwiększały TAC osocza u szczurów z uszkodzeniami wątroby wywołanymi przez chloropiryfos. **Wnioski:** Badanie wykazało, że CAPE i CAPE+IL poprzez zmniejszenie stresu oksydacyjnego i apoptozy redukują u szczurów uszkodzenia wątroby wywołane przez chloropiryfos. *Med. Pr.* 2016;67(6):743–749

Słowa kluczowe: apoptoza, stres oksydacyjny, wątroba, szczury, chloropiryfos, ester fenetylowy kwasu kawowego

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INTRODUCTION

Chlorpyrifos (CPF), an organophosphate (OP) compound, is commonly used against insects in agriculture and on animal farms [1]. However, indiscriminate applications of the pesticides may result in environmental pollution and human and animal poisonings [2]. Organophosphate induced poisonings may occur by inhalation, cutaneous absorption, or ingestion [3]. Inhibition of acetylcholinesterase activity and accumulation of excess acetylcholine at the nerve endings may lead to muscarinic and nicotinic clinical signs [2,4]. Toxic clinical signs induced by OP poisonings may also develop through the occurrence of oxidative stress [5,6]. Several studies have reported that OP compounds may cause various organ injuries and oxidative stress [3,7–9].

Caffeic acid phenethyl ester (CAPE) is one of active components of propolis that is a honey bee product [10]. Traditional medicine takes advantage of propolis as a dietary supplement for therapeutic aims. It has neuroprotective, hepatoprotective and cardioprotective activities with anti-inflammatory and anti-oxidative effects [11,12].

Approved for parenteral nutrition, intravenous lipid emulsion (ILE) has become a focus of interest in the treatment of lipophilic drug toxicities [13]. Intravenous lipid emulsion has been reported to successfully treat the toxicity of local anesthetics, calcium channel blockers, β -blockers, and a variety of psychotropic agents [14,15]. However, the mechanism of ILE action has not yet been explored thoroughly [15]. Further research still remains to be conducted in order to discover whether or not ILE is safe to use for treating toxicity from chlorpyrifos.

The need for additional research concerning the possibility of OP poisoning is also important, especially in view of the fact that 3 million acute poisoning cases are reported worldwide every year [16,17]. The aim of this study has been to evaluate the effects of caffeic acid phenethyl ester and intralipid on hepatotoxicity induced by chlorpyrifos in the case of rats.

MATERIAL AND METHODS

Animals

The current study was approved by Necmettin Erbakan University and the Experimental Animal Ethics Committee in Konya, Turkey, with the ethic No. 2012/057. The animal material consisted of 49 adult Wistar albino rats, each weighing 200–250 g. They were kept in stand-

ard housing facilities at room temperature of $21\pm 2^\circ\text{C}$, in a 12-h light–dark cycle. The experimental protocol was carried out according to the International Guidelines for the Care and Use of Laboratory Animals.

Experimental process

The rats were randomly divided into 7 groups of 7 rats each, as follows:

1. The control group.
2. The chlorpyrifos (CPF) group was treated with CPF 10 mg/kg body weight (b.w.), orally [4].
3. The intralipid (IL) group was treated with IL 18.6 ml/kg b.w., orally [18].
4. The caffeic acid phenethyl ester (CAPE) group was treated with CAPE 10 $\mu\text{mol/kg}$ b.w., intraperitoneally [19].
5. The CPF+IL group was treated with CPF and IL.
6. The CPF+CAPE group was treated with CPF and CAPE.
7. The CPF+CAPE+IL group was treated with CPF, CAPE, and IL.

The single dose treatments were made with CPF, IL, and CAPE. The rats were treated with CPF, immediately followed by IL and/or CAPE.

Sample collection

The rats were sacrificed using ketamine (50 mg/kg b.w., intraperitoneal (i.p.), Ketalar, Parke Davis, Turkey) and xylazine (5 mg/kg b.w., i.p., Rompun, Bayer AG, Germany) immediately after the administration of the drugs. The plasma samples of the rats had been removed, centrifuged and stored at -70°C until they were used for biochemical analysis. The liver tissues were stored in a formaldehyde solution for histopathological examination purposes.

Total antioxidant capacity (TAC) and total oxidant capacity (TOC) analysis

The TAC and TOC levels of plasma samples were measured by a commercial test kit (Rel Assay Diagnostics, Turkey). The TAC was measured by a novel automated and colorimetric method developed by Erel [20]. In this method, mixing reagent 1 (xylenol orange 150 μM , NaCl 140 mM and glycerol 1.35 M in 25 mM H_2SO_4 solution, pH 1.75) and reagent 2 (ferrous ion 5 mM and o-dianisidine 10 mM in 25 mM H_2SO_4 solution) produced hydroxyl radicals, which converted o-dianisidine molecules into dianisidyl radicals, resulting in a bright yellow-brown color. After adding the supernatant, the antioxidants of the supernatant suppressed

further color development. The TAC in the supernatant was measured spectrophotometrically, with lower than 3% precision values. The data of the TAC is expressed as mmol Trolox equivalent/l for the plasma. Erel's method [21] was also used for measuring the plasma TOC levels.

The conversion of the ferrous ion-o-dianisidine complex to a ferric ion was provided by the oxidants of the supernatant; glycerol molecules of the reaction medium conducted this oxidation reaction. A colored complex with xylenol orange was produced by the ferric ion. The oxidant molecules in the supernatant were measured spectrophotometrically with color intensity. The data of the TOC is expressed as mmol H₂O₂ equivalent/l for plasma. The calculation of the oxidative stress index (OSI) was made by the percent ratio of the TOC to the TAC (OSI (arbitrary unit) = TOC (mmol H₂O₂ equivalent/l)/TAC (mmol Trolox equivalent/l)) [9].

Histopathological analysis

The fixed liver tissues were embedded in paraffin. Four μ m-thick sections were cut from paraffin blocks using microtome (Leica Rotary, Leica Microsystems GmbH, Germany), stained with hematoxyline and eosin (HE) and examined under a light microscope ($\times 100$). The liver tissue injury was scored 0–3: with none or mild changes (0); mild cytoplasmic vacuolization, and nuclear pyknosis (1); moderate nuclear pyknosis, cytoplasmic hypereosinophilia, and intercellular adhesion loss (2); severe hemorrhage, neutrophil infiltration, intercellular adhesion loss, and necrosis (3).

Immunohistochemistry

The immunohistochemistry process was performed using a Leica Bond-Max automated IHC/ISH platform (Leica Microsystems Inc., USA). The liver sections were dewaxed in a Bond Dewax solution and rehydrated in alcohol and a Bond Wash solution (Leica Microsystems Inc., USA). Retrieval of the antigen was carried out using a high pH (ER2) retrieval solution for 15 min; next, the sections were blocked using peroxidase for 5 min on the machine. An anti-mouse monoclonal antibody Bcl-2 (C-2, sc-7382 (Santa Cruz Biotechnology Inc., USA) in dilution 1:200), an anti-mouse monoclonal antibody Bax (B-9, sc-7480 (Santa Cruz Biotechnology Inc., USA) in dilution 1:100), and an anti-mouse caspase 3 (CPP32) monoclonal antibody (clone JHM62 (Leica Biosystems Ltd., Great Britain) in dilution 1:50) were applied for 60 min at room temperature. Detection

was complied with using the Bond Polymer Refine Red Detection system (Leica Microsystems GmbH, Germany). The liver apoptosis was evaluated in 10 randomly selected microscope fields under $\times 400$ magnification.

Statistical analysis

The statistical analysis of the data in the Statistical Package for the Social Sciences (SPSS) package program (version 12, SPSS, USA) was performed by means of a one-way ANOVA. *Post-hoc* Tukey and Dunnett T3 tests were used for comparing the studied groups. The differences were considered significant if $p < 0.05$.

RESULTS

TAC levels

There was no significant difference in the plasma TAC level between the control group and the IL, CAPE, CPF+CAPE, and CPF+CAPE+IL groups. The plasma TAC level in the CPF group was significantly decreased in comparison to that of the control, IL, CAPE, CPF+CAPE, and CPF+CAPE+IL groups ($p < 0.05$). There was no significant difference in the TAC level between the CPF group and the CPF+IL group (Table 1).

TOC levels

The plasma TOC level was significantly increased in the CPF group as compared to the control, IL, CAPE, CPF+CAPE, and CPF+CAPE+IL groups ($p < 0.05$). There was no significant difference in the TOC level between the control group and the IL, CAPE, CPF+IL, CPF+CAPE, and CPF+CAPE+IL groups (Table 1).

OSI levels

There was no significant difference in the OSI level between the control group and the IL, CAPE, CPF+CAPE, and CPF+CAPE+IL groups. The plasma OSI level was significantly increased in the CPF and CPF+IL groups as compared to the control group ($p < 0.05$). There was no significant difference in the plasma OSI level between the CPF group and the CPF+IL group, and there was a significant difference in the OSI level between the CPF group and the control, IL, CAPE, CPF+CAPE, and CPF+CAPE+IL groups ($p < 0.05$) (Table 1).

Histopathological evaluation

Nuclear pyknosis, cytoplasmic hypereosinophilia, cytoplasmic vacuolization, and intercellular adhesion loss were not observed in the control (Photo 1a),

Table 1. Effects of caffeic acid phenethyl ester (CAPE) and intralipid (IL) on hepatotoxicity of chlorpyrifos (CPF) in rats
Tabela 1. Wpływ estru fenetylowego (CAPE) i intralipidu (IL) na hepatotoksyczność chloropiryfosu (CPF) u szczurów

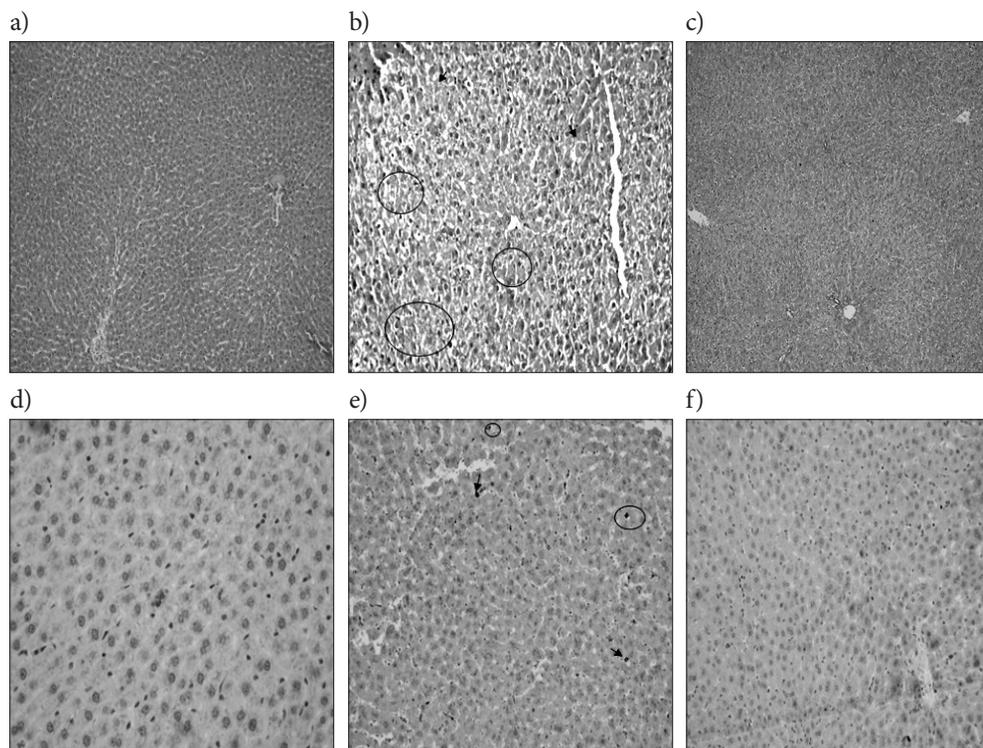
Parameter Parametr	Study group Grupa badana						
	control kontrolna (N = 7)	CPF (N = 7)	IL (N = 7)	CAPE (N = 7)	CPF+IL (N = 7)	CPF+CAPE (N = 7)	CPF+CAPE+IL (N = 7)
TAC [mmol/l] (M±SEM)	1.00±0.04	0.66±0.02 ^a	1.04±0.02	1.11±0.04	0.69±0.01 ^a	0.83±0.03	0.96±0.04
TOC [mmol/l] (M±SEM)	43.06±2.62	58.82±3.85 ^a	43.26±1.90	38.51±2.29	54.87±2.90	46.88±2.98	46.40±2.44
OSI (TOC/TAC) (M±SEM)	42.98±1.79	89.64±6.76 ^a	41.65±1.82	34.73±1.91	79.44±4.37 ^a	56.80±5.06	48.37±2.94
HP (M±SEM)	3.14±0.50	14.85±1.14 ^a	6.00±0.30	4.00±0.21	11.42±0.71 ^a	8.28±0.74	8.42±0.64
IHC (M±SEM)	16.71±1.30	145.42±2.75 ^b	15.14±0.79	13.42±0.71	127.00±1.79	78.85±1.38	66.00±2.71

TAC – total antioxidant capacity / całkowita zdolność przeciwutleniająca, TOC – total oxidant capacity / całkowita zdolność utleniająca, OSI – oxidative stress index / wskaźnik stresu oksydacyjnego, HP – histopathology / badanie histopatologiczne, IHC – immunohistochemistry / badanie immunohistochemiczne.

M – mean / średnia, SEM – standard error of the mean / błąd standardowy średniej.

^a p < 0.05 vs. the control, IL, CAPE, CPF+CAPE, and CPF+CAPE+IL group / p < 0,05 vs grupa kontrolna, IL, CAPE, CPF+CAPE i CPF+CAPE+IL.

^b p < 0.05 vs. the control, IL, CAPE, CPF+IL, CPF+CAPE, and CPF+CAPE+IL group / p < 0,05 vs grupa kontrolna, IL, CAPE, CPF+IL, CPF+CAPE i CPF+CAPE+IL.



a) control group – normal liver histology (image magnification $\times 200$) / grupa kontrolna – wynik badania histologicznego wątroby w normie (powiększenie obrazu $\times 200$), b) CPF group – severe liver damage and necrosis (indicated by the arrows and circles) ($\times 200$) / grupa CPF – poważne uszkodzenia i martwica wątroby (zaznaczone strzałkami i kółkami) ($\times 200$), c) CPF+CAPE+IL group – regressive regeneration including regenerative changes suggestive of normal liver histology and focal cytoplasmic hyper eosinophilia ($\times 200$) / grupa CPF+CAPE+IL – regresywna regeneracja, w tym zmiany regeneracyjne wskazujące na wynik badania histologicznego wątroby w normie i ogniskową cytoplazmatyczną hipereozynofilię ($\times 200$), d) control group – immunoreactivity as opposed to the control group ($\times 200$) / grupa kontrolna – immunoreaktywność w porównaniu z grupą kontrolną ($\times 200$), e) CPF group – immunoreactivity suggesting increased apoptosis in the liver tissues (indicated by the arrows and circles) ($\times 400$) / grupa CPF – immunoreaktywność wskazująca na zwiększoną apoptozę w tkankach wątroby (zaznaczoną strzałkami i kółkami) ($\times 400$), f) CPF+CAPE+IL group – apoptosis level was only lower than the one in the CPF group ($\times 400$) / grupa CPF+CAPE+IL – poziom apoptozy niższy tylko w porównaniu z grupą CPF ($\times 400$).

Abbreviations as in Table 1 / Skróty jak w tabeli 1.

Photo 1. Liver tissues of rats studied under light microscope (a–c) using hematoxyline and eosin (HE) stain and (d–f) immunohistochemical (IHC) method

Fot. 1. Tkanki wątroby szczura badane pod mikroskopem optycznym (a–c) po barwieniu hematoksyliną i eozyną (HE) (d–f) po zastosowaniu metody immunohistochemicznej (IHC)

IL, and CAPE groups. Severe hemorrhage, neutrophil infiltration, nuclear pyknosis, cytoplasmic hyper-eosinophilia, cytoplasmic vacuolization, intercellular adhesion loss, and necrosis were observed in the CPF group (Photo 1b). These severe hemorrhage, neutrophil infiltration, intercellular adhesion loss, and necrosis changes were significantly decreased in the CPF+CAPE and CPF+CAPE+IL groups as compared to the CPF group (Table 1, $p < 0.05$, Photo 1c). There was no significant difference in these changes between the CPF group and the CPF+IL group.

Immunohistochemistry evaluation

Immunohistochemistry showed significantly increased apoptosis in the liver tissues of the CPF group as compared to the control group (Photo 1d and 1e) and significantly decreased apoptosis in the CPF+CAPE and CPF+CAPE+IL groups as compared to the CPF group. The significant decrease in the apoptosis was determined in the CPF+CAPE+IL group (Photo 1f). The apoptosis decrease in the CPF+CAPE group and the CPF+CAPE+IL group was significantly higher than in the CPF+IL group ($p < 0.05$).

DISCUSSION

Organophosphates cause oxidative stress by altering the balance between the production of oxidants and antioxidants [2]. The chlorpyrifos exposure in the case of rats has been reported to cause peroxidative damage in various tissues by enhancing lipid peroxidation products [22]. In this study, the plasma TOC and OSI levels in the case of rats were significantly increased in the CPF group as compared to the control group. This result suggests that a single oral dose of the CPF (10 mg/kg b.w.) induces oxidative stress in the plasma of rats. The oxidative stress induced by rats' exposure to the CPF in this study has also been reported in other similar studies carried out for mice or rats [7,22–24].

The mechanism of OP compounds that produce oxidative stress has been involved with their metabolism by the cytochrome P450 [22]. To counteract the oxidative stress, the plasma TAC levels regarding the acute CPF toxicity of the CPF group in the case of rats have been found to significantly decrease as compared to the control group. In the event of the CPF induced toxicity, various studies have found that antioxidant activity increases or decreases depending on the amounts of the CPF doses and the durations of the treat-

ment [3,16,23]. The decreased TAC levels induced by the acute CPF toxicity may suggest the consumption of the TAC due to significantly increased plasma OSI levels.

Research continues to be conducted to find a way to reduce the oxidative stress responsible for the pathogenesis of OP compounds. For this purpose, an anti-inflammatory agent such as rosiglitazone [25], antioxidants agents such as vitamin C [23], N-acetylcystein [26], melatonin [27], quercetin [8], pomegranate, and rutin [28] have been reported as the attenuating agents for CPF induced toxicity.

For the first time has this study revealed the attenuating effects of the CAPE and CAPE+IL treatment on oxidative stress and apoptosis in the CPF induced liver toxicity of rats. Caffeic acid phenethyl ester is known to have neuroprotective, hepato-protective, and cardio-protective activities with anti-inflammatory and anti-oxidative effects [11,12,29], and the ILE has become a focus of interest in the treatment of lipophilic drug toxicities [15]. Caffeic acid phenethyl ester and the CAPE+IL have been reported to be effective in attenuating the renal toxicity induced by dichlorvos that is an OP compound [9]. In this study, the CAPE and CAPE+IL treatment provided a significant increase in the plasma TAC levels against the CPF induced liver toxicity. However, the IL alone could not significantly increase the plasma TAC levels in the CPF toxicity of rats.

Membrane lipids, membrane receptors, and membrane-bound enzymes may be affected by lipid peroxidation and by altering the membrane function, fluidity, and structure [30]. Organophosphates may induce injuries in various organs and cells [3,7–9,24]. The exhibited histopathological changes due to the CPF induced liver toxicity were attenuated by the CAPE and CAPE+IL treatment, but not by exclusively the IL treatment.

The chlorpyrifos induced cellular toxicity leads to oxidative stress [25], caspase-3 activation and subsequent increased release of cytochrome c into the cytosol [26]. Mitochondrial dysfunction induces oxidative stress, deoxyribonucleic acid (DNA) damage, and apoptosis [26].

CONCLUSIONS

This study revealed that cell death caused by the CPF in the liver of rats is involved in apoptosis through increased OSI levels and activated caspase-3. Caffeic acid phenethyl ester and the CAPE+IL treatment attenuated apoptosis by inhibiting caspase-3 activation in the rats with exposure to the CPF. The most effective

caspase-3 inhibition was provided by the CAPE+IL treatment. This study suggests that the CAPE and CAPE+IL treatment may attenuate the CPF induced liver injury by decreasing oxidative stress and inhibiting caspase-3 activation.

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