Protective role of caffeic acid phenethyl ester on serum cholinesterase inhibition by acute exposure to diazinon in rats

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Aim: To evaluate whether caffeic acid phenethyl ester (CAPE), a flavonoid-like natural compound plentifully found in beeswax, has a protective effect on diazinon-induced serum cholinesterase (ChE) inhibition in rats.

Materials and methods: Animals were divided into 4 groups. The first animal group was not treated with any substance. The second animal group was orally given a 200 mg/kg body weight (bw) sublethal dose of diazinon. The third animal group was injected intraperitoneally with 2.84 mg (10 µmol)/kg bw of CAPE 1 day prior to administration of 200 mg/kg bw of diazinon orally. The fourth animal group was intraperitoneally injected with 2.84 mg (10 µmol)/kg bw of CAPE 30 min after 200 mg/kg bw of diazinon was orally administered.

Results: Analysis of the animal blood samples obtained 48 h after diazinon administration revealed that diazinon decreased serum ChE activity by 75%, while CAPE administration 24 h prior to and 30 min following diazinon application improved serum ChE activity by 25%–32% as compared to levels with diazinon administration only. In silico studies suggest that CAPE prevents diazinon from binding to butyryl ChE due to a higher binding affinity than that of diazinon.

Conclusion: Our laboratory findings suggest that CAPE plays a protective role against butyryl ChE inhibition by diazinon.

Key words: Organophosphate, diazinon, caffeic acid phenethyl ester, serum cholinesterase, docking

1. Introduction
Organophosphate insecticides (OPI) such as diazinon (Figure 1) are among the most commonly used compounds worldwide, and are also taken orally by those committing suicide in countries dealing with agriculture (1,2). They inactivate cholinesterase (ChE) by irreversibly binding to a catalytic serine amino acid residue in the active site of the enzyme. This in turn impedes the hydrolysis of excess acetylcholine (ACh), which results in deposition of ACh at the neuromuscular junctions disabling neurotransmission to the muscles. If neurotransmission to the respiratory muscles is incapacitated by OPIs, death is not unavoidable if mechanical ventilation and other supportive measures are provided (3).

Anyone who is exposed to OPIs, such as farmers dealing with OPIs or workers engaged in the production of organophosphate (OP) compounds, should regularly go for blood tests to monitor changes in the ChE levels before serious symptoms occur. Human blood has 2 types of ChE: 1) red blood cell or erythrocyte cholinesterase (EChE), called true cholinesterase or AChE; and 2) serum ChE (SChE), called pseudocholinesterase or butyrylcholinesterase (BChE) (4). EChE is the same enzyme that is found in the nervous system, lungs, and spleen, while SChE is made in the liver and also found in the pancreas. SChE readings are helpful for detecting the early, acute effects of OP poisoning, while EChE readings are taken into account for long-term or chronic exposure. In general, blood ChE activity drops by 15%–25%, 25%–35%, and 35%–50% upon low, moderate, and severe intoxication by pesticides, respectively (5). The main treatment for OPI poisoning is the application of atropine as an ACh receptor blocker. In addition, oxime-like compounds such as pralidoxime and obidoxime (Figure 1) are also used as antidotes to prevent OPI from chemically modifying a catalytic serine amino acid residue in the active site of ChE (6).
It has been previously determined that caffeic acid phenethyl ester (CAPE) (Figure 1) plays a substantial role in ameliorating isoniazid-induced oxidative damage to red blood cells in rats (7), cadmium-induced cardiac impairment in rats (8), and cisplatin-induced oxidative damage to the rat liver (9), as well as controlling superoxide dismutase stress enzyme activities in diabetic rat retinas (10) and inducing apoptosis in a dose- and time-dependent manner in human multiple myeloma cells (11). However, studies on the effect of CAPE on SChE activity in the presence of OPIs have not been satisfactorily documented in the literature. Thus, the main objective of this work is to provide more insight into the effect of CAPE on SChE in the presence of diazinon, an OPI, by animal as well as in silico studies, and to discuss the potential application of CAPE as an alternative or perhaps complementary antidote in ameliorating the early or acute phase of SChE inhibition by diazinon.

2. Materials and methods

2.1. Chemicals

CAPE was obtained from Sigma-Aldrich (St. Louis, MO, USA). Diazinon (Basudin*) was obtained from Syngenta (Izmir, Turkey). Ketamine (Ketalar*) was obtained from Eczacibaşı (İstanbul, Turkey). Xylazine (Rompun*) was obtained from Bayer (İstanbul, Turkey).

2.2. Animal studies

All animal experiments were approved by the Experimental Animal Ethics Committee of the Health Science Research and Application Center at Süleyman Demirel University in Isparta, Turkey. All animal studies were conducted in accordance with the laws and regulations of the governing authorities. Animal studies were carried out using 35 young Wistar albino male rats. The rats were divided into 4 groups. The control group of rats (Group I, n = 5) was orally given corn oil only via orogastric tube. The second group of rats (Group II, n = 10) was orally given a sublethal dose of 200 mg/kg body weight (bw) of diazinon (12) in corn oil, which was prepared by dissolving a required amount of Basudin in a solution of 60 mg/mL diazinon in corn oil. The third group of rats (Group III, n = 10) were intraperitoneally (ip) given 2.84 mg (10 µmol)/kg bw of CAPE 1 day prior to administration of 200 mg/kg bw of diazinon, dissolved in corn-oil and orally applied via orogastric tube (13). The fourth group of rats (Group IV, n = 10) were ip injected with 2.84 mg (10 µmol)/kg bw of CAPE 30 min administration of 200 mg/kg bw of diazinon, dissolved in corn oil and administered via orogastric tube.

2.3. Biochemical analysis

All animals received general anesthesia ip by administration of 50 mg/kg bw of ketamine and 1 mg/kg bw of xylazine 48 h after diazinon treatment. Animals under general anesthesia were surgically decapitated and cut open with an incision of the median line, and 4 mL of blood samples were collected from the vena cava inferior of each incised animal for biochemical analysis. Serum samples were prepared by the centrifugation of the blood samples at 4000 rpm. Cholinesterase activities in the serum samples were measured with an Architect c16000 Abbott Aeroset® instrument (Abbott Laboratories, Abbott Park, IL, USA), which applied BCh as the substrate for serum BChE to yield butyrate and thiocholine as the hydrolysis products. Thiocholine then reduces hexacyanoferrate(III) to hexacyanoferrate(II). A decrease in the absorbance of hexacyanoferrate(III) is directly proportional to the ChE activity in the sample.

2.4. In silico studies

Docking studies were carried out with AutoDock Vina v.1.1 (14). The X-ray structure of human BChE in complex with a tabun molecule, a known inhibitor of the substrate binding site of the enzyme, was obtained from the Protein Data Bank, PDB ID: 2WID (15). The X-ray structure of aged human AChE, in which the catalytic SER200 is phosphoramidylated, in complex with snake venom fasciculin-II was obtained from the Protein Data Bank, PDB ID: 2X8B (16). All nonprotein molecules including tabun, phosphoramidite, fasciculin-II, water, and ions were removed from PDB structures before docking. Docking of ligands, including CAPE, pralidoxime, diazinon, ACh,
and BCh, was implemented in a confined grid-box defined by MGL Tools v.1.5.4 (17). Docked ligands were analyzed by MGL Tools v.1.5.4. The binding cavity volume of the receptor species was computed by the online version of the POCSASA v.1.0 software (18). Molecular surface volume of the ligands was determined by the MSMS module available in UCSF Chimera v.1.4.1 (19).

2.5. Statistical analysis
Mean ChE activity and standard deviations (SDs) were computed using serum ChE activity data obtained from the control group and the animals that survived diazinon administration. The data groups were compared by one-way ANOVA statistical analysis. Analysis of variance was determined by Tukey’s honestly significant difference post hoc analysis.

3. Results
From a clinical point of view, it was observed that the clinical status of some animals poisoned by diazinon in Groups II, III, and IV deteriorated, with the animals exhibiting severe symptoms of diarrhea, eye rash, abdominal distention, and immobility; clinical conditions culminated in death due to diazinon administration. Four animals in Group II, to which only diazinon was administered; 3 animals in Group III, to which CAPE was given prior to diazinon administration; and 4 animals in Group IV, to which CAPE was given following diazinon administration, were reported dead (Table 1).

Mean values for serum ChE activities, expressed as U/L, and their statistical deviations as referenced to the control group (Group I) and the surviving animals of Group II are given in Table 1. In addition, the serum ChE activities determined for each surviving animal are plotted in Figure 2. Percent activities for Group II, III, and IV animals were referenced to the activity of Group I animals, which was set to 100% in Table 1. As seen in Table 1, the mean serum ChE activity of Group II animals (22%) was significantly lower than that of Group I animals (100%). It was also observed in Group III (50%) and Group IV (43%) that serum ChE activities improved as compared to Group II by 25% to 32% upon administration of CAPE prior to and following diazinon application, respectively (Table 1).

Binding free energies, ΔG°, as a sum of electrostatic and Van der Waals energies in the gas phase determined by AutoDock Vina v.1.1 (14), are given in Table 2 for the most likely bound conformations of ACh, BCh, diazinon, pralidoxime, and CAPE in the Ser198 catalytic binding site of BChE. Three binding conformations of CAPE with binding energies ΔG°1 = −8.2 kcal/mol, ΔG°2 = −7.9 kcal/mol, and ΔG°3 = −7.2 kcal/mol, respectively; 2 binding conformations of pralidoxime with binding

<table>
<thead>
<tr>
<th>Clinical status (dead/surviving)</th>
<th>% ChE activity</th>
<th>Mean ChE activity (U/L ± SD)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>n = 5</td>
<td>0/5</td>
<td>100</td>
</tr>
<tr>
<td>Group II</td>
<td>n = 10</td>
<td>4/6</td>
<td>22</td>
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<tr>
<td>Group III</td>
<td>n = 10</td>
<td>3/7</td>
<td>50</td>
</tr>
<tr>
<td>Group IV</td>
<td>n = 10</td>
<td>4/6</td>
<td>43</td>
</tr>
</tbody>
</table>

*: Significance between Group II animals (surviving) and the other groups’ animals (surviving).
†: Statistically significant change in experimental values of Group III animals (surviving) as compared to Group II animals (surviving) (P = 0.002).
‡: Statistically significant change in experimental values of Group IV animals (surviving) as compared to Group II animals (surviving) (P = 0.023).
energies of $\Delta G^\circ_1 = -5.7$ kcal/mol and $\Delta G^\circ_2 = -5.7$ kcal/mol, respectively; 1 binding conformation of diazinon with a binding energy of $\Delta G^\circ = -6.7$ kcal/mol; 1 binding conformation of BCh with a binding energy of $\Delta G^\circ = -5.2$ kcal/mol; and 1 binding conformation of ACh with a binding energy of $\Delta G^\circ = -4.4$ kcal/mol were determined in the crystal structure of the active site of BChE. Table 2 also lists the corresponding association binding constants determined by $\Delta G^\circ = -RT \ln K_a$, where $R$ is 1.987 cal/(mol K) and $T$ is 298.15 K, and the volumes are in Å$^3$ for the binding site and the ligands. Docking binding energies and the corresponding association constants indicate that CAPE exhibits the greatest affinity towards the Ser198 catalytic binding site of BChE.

In addition, docking studies were carried out for the binding site of human BChE; results are shown in Table 3 for diazinon, ACh, and CAPE. CAPE has only 2 binding modes as compared to the 3 binding conformations for BChE seen in Table 2. This is because AChE possesses a narrower binding cleft than that of BChE and, therefore, CAPE samples have fewer binding conformations in the binding site of AChE. CAPE binding poses gave rise to the highest binding affinities with binding energies of $\Delta G^\circ = -9.1$ kcal/mol and $\Delta G^\circ = -8.3$ kcal/mol. The binding energy of diazinon in the binding site of BChE was found to be $\Delta G^\circ = 7.4$ kcal/mol, which means a greater affinity than that of ACh at $\Delta G^\circ = -5.0$ kcal/mol.

4. Discussion
It was found in our laboratory via animal and computational experiments that CAPE, shown in Figure 1, exhibits a protective effect against SChE inhibition by diazinon. In terms of public health, CAPE is quite safe to use as it is a natural product found primarily in beeswax and it causes much less side effects than a synthetic drug (7). It was determined by animal experiments that diazinon severely reduced SChE activity by 75% as it was given to rats; see the results for Group II in Table 1 and Figure 2.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Conformation</th>
<th>$\Delta G^\circ$ (kcal/mol)</th>
<th>$K_a$ (L/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACh</td>
<td>I</td>
<td>-5.0</td>
<td>$4.6 \times 10^3$</td>
</tr>
<tr>
<td>Diazinon</td>
<td>I</td>
<td>-7.4</td>
<td>$2.7 \times 10^4$</td>
</tr>
<tr>
<td>CAPE</td>
<td>I</td>
<td>-9.1</td>
<td>$4.7 \times 10^6$</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>-8.3</td>
<td>$1.2 \times 10^7$</td>
</tr>
</tbody>
</table>

N/A = not applicable.
In the next set of animal experiments, as is observed in Table 1 and Figure 2, SChE activity decreased by 50% in Groups III and IV when CAPE was ip injected prior to and following oral administration of diazinon. The reason for the application of CAPE prior to diazinon intake was to see if CAPE would protect the rats from diazinon poisoning, while CAPE application following diazinon intake aimed to see its protecting capability in the case of diazinon poisoning. As a result, it was determined in these animal experiments that CAPE increased SChE activity by 25% upon diazinon application.

Looking at Table 1 for the number of dead animals in Groups II, III, and IV, it can be said that CAPE is best able to protect SChE from the toxic effects of diazinon if it is applied prior to exposure to OP diazinon, as in the case of the Group III animals.

The binding free energies given in Table 2 strongly suggest that CAPE binds to human BChE with the greatest affinity as compared to other compounds listed in Table 2, making it a precautionary natural substance, even at low-dosage administrations, to diminish possible inhibition of SChE by OP compounds.

Molecular surface volumes for the most favorable docked coordinates of CAPE and ACh are superimposed in the catalytic binding site of human BChE in Figure 3. As seen in Table 2, surface volumes for CAPE and ACh are ~260 Å³ and 148 Å³, respectively, which can be comfortably accommodated in the binding site of BChE, and are shown in magenta and cyan, respectively, in Figure 3. As diazinon possesses a volume of 275 Å³, larger than that of ACh, it becomes more difficult to accommodate CAPE and diazinon together in the binding site. It is highly likely that the occupation of CAPE in the catalytic site of BChE prevents diazinon from interacting with Ser198 and yet maintains the activity of the enzyme at the minimum levels until ACh accumulates up to competitive concentrations, at which the enzymatic hydrolysis reaction with ACh takes place.

It should be noted that this paper merely evaluates the activity of SChE in the presence of diazinon. SChE is mainly composed of BChE, which is used as a biomarker of exposure to OPs. This paper does not evaluate OP-induced respiratory muscle impairment at neuromuscular junctions due to AChE inhibition, which would be indirectly measured as EChE activity in red blood cells (20), a biomarker of measuring AChE activity in the nervous system. Therefore, the empirically determined SChE activities presented in Table 1 should not be correlated to AChE inhibition in the nerve tissue.

SChE is more labile than EChE and, thus, SChE activity would drop the most rapidly in response to exposure to an OPI, making SChE a good and cost-effective biomarker of acute OPI poisoning. As the aim of this work was to monitor only the acute effects of exposure to OPI in the short-term, not the chronic effects of exposure to OPI in the long-term, only SChE activities were tested in animals.

To gain more structural insight into human ChE enzymes, the crystal structures of human BChE [PDB ID: 2WID (15)], white in Figure 4, and of human AChE [PDB ID: 3LI2 (21)], red in Figure 4, were superposed, which gave rise to a 52.36% sequence identity and a high degree of structural similarity in the binding site. In particular, the locations of the catalytic histidine (HIS) and serine (SER) amino acid residues are well preserved in the binding sites of human BChE and human AChE. Furthermore, the docking of ACh, diazinon, and CAPE into the binding site of human AChE revealed that CAPE...
binds with greater affinities than does diazinon (Table 3). Indeed, it was computationally found that CAPE binds to AChE with greater affinity than it does to BChE; see Tables 2 and 3 for CAPE's association constants.

In silico findings strongly suggest that human AChE and BChE are structurally very similar and that it is highly likely that CAPE protects AChE inhibition by OPs in the nerve cells. However, in order to support such a putative conclusion, further animal and biochemistry experiments are in progress to monitor EChE activities in presence of OPs.

In conclusion, the animal, biochemistry, and in silico results presented in this paper suggest that CAPE exhibits the best protective effects against BChE inhibition by OPs if it is applied prior to exposure to OP. Therefore, it is highly recommended that natural beeswax or CAPE itself be used as a precautionary natural antidote by farmers and workers prior to dealing with OPs in order to diminish the acute effects of SChE inhibition; however, the use of CAPE requires further safety studies.

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