Original article

3-nitrotyrosine levels in dichlorvos-induced neurotoxicity

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The aim of this study was to evaluate dichlorvos toxicity in terms of nitro-oxidative stress by determining 3-nitrotyrosine (3-NT) levels in the fore, mid, and hindbrain regions in acutely exposed rats. Male Sprague-Dawley rats were randomly allocated to three groups of eight. Group 1 was administered a single intraperitoneal dichlorvos dose of 1.8 mg kg^{-1} ($0.1 \times LD_{50}$) and group 2 a dose of 9 mg kg^{-1} ($0.5 \times LD_{50}$). The control group received 0.5 mL saline solution via the same route. 3-NT and tyrosine (TYR) levels were measured using high performance liquid chromatography with a photodiode array detector (HPLC-PDA) and expressed as a ratio of 3-NT to TYR. The 3-NT/1000 TYR ratios increased significantly in the fore-, mid- and hindbrains of the exposed groups compared to control ($p<0.01$). In the forebrain, the increase was also significant between the treated groups. Our study has confirmed that acute exposure to dichlorvos leads to nitro-oxidative stress in the brain and that 3-NT may play a role in the mechanism of dichlorvos neurotoxicity.

KEY WORDS: *brain; HPLC; nitro-oxidative stress; pesticides; rats*

Dichlorvos (DDVP-2,2-dichlorovinyl dimethyl phosphate) is a pesticide commonly used for the protection of stored products and grains, for controlling ecto- and endoparasites of farm animals, and for combating indoor and outdoor pests. Its annual world production reaches four thousand tonnes (1). Being an organophosphorous compound, its principal mechanism of toxicity is through the inhibition of acetylcholinesterase (AChE) and/or neuropathy target esterase (NTE), which acts on the central and peripheral nervous systems (2).

In addition, dichlorvos toxicity seems to induce oxidative stress (3, 4), whose product peroxynitrite (5) may react with various amino acid residues in proteins (6). The product of tyrosine nitration is 3-nitrotyrosine (3-NT), and it is often used as a biomarker of oxidative attack on biological molecules (6, 7). However, research evaluating OP-induced oxidative stress in terms of 3-NT levels is limited. The aim of our study was to contribute to the scarce information about 3-NT measurements in the brain and to see how dichlorvos affects its levels through oxidative stress.

MATERIALS AND METHODS

Reagents

Analytical grade chemicals, standards, and solvents were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Animals and treatment

Prior to the experiment, all the experimental procedures were approved by the Experimental Animal Studies Ethics Committee of Ondokuz Mayis University in Samsun, Turkey.

Twenty-four male Sprague-Dawley rats (250-300 g), maintained in a climate-controlled room at 22±2 °C on a 12 h light/dark cycle and receiving standard pellet diet and water *ad libitum*, were

randomly allocated to three groups of eight animals. Group 1 received a single intraperitoneal (*i.p.*) dose of 1.8 mg kg^{-1} (0.1xLD$_{50}$) of dichlorvos, group 2 9 mg kg^{-1} (0.5xLD$_{50}$), and the control group received 0.5 mL of saline solution via the same route (1). No rat showed visual signs of toxicity or died because of treatment. Three days after dichlorvos or saline administration, the rats were killed by cervical dislocation and brain specimens stored at -80 °C until analysis.

Sample preparation

3-NT levels were expressed as μmol L^{-1} per 1000 μmol L^{-1} of tyrosine (3-NT/1000 TYR) to normalise for differing brain concentrations of tyrosine (8, 9). Calibration curves were produced for 3-NT and TYR. The standard calibration curves were linear over a concentration range of 1-8 μmol L^{-1} and 100-1000 μmol L^{-1} for 3-NT and TYR, respectively. Frozen brain samples were sonicated in 10 mmol L^{-1} of cold sodium acetate at pH 6.5 and protein concentrations were determined using the Bradford method (10). Homogenised brain samples (5 mg mL^{-1}) were centrifuged at 14,000 *g* and 4 °C for 10 min. The supernatant was collected and treated with 10 mg mL^{-1} Pronase (*Streptomyces griseus* protease) at 50 °C for 18 h. After incubation, samples were treated with a 10 % volume of 60 % of trichloroacetic acid (TCA) and centrifuged at 14,000 *g* and 4 °C for 10 min. The supernatant was removed and passed through a 0.2 μm polyvinylidene difluoride (PVDF) membrane, and the filtrate was analysed with high performance liquid chromatography with a photodiode array detector HPLC-PDA (11-13).

High-performance liquid chromatography (HPLC)

The HPLC system (Shimadzu, LC-20AT Prominence, Kyoto, Japan) consisted of a pump, an auto-injector (SIL-20AC), and a diode array detector (SPD-M20A). 3-NT and TYR were separated in a 5 μm, 4.6x250 mm C$_{18}$ reverse phase (Inertsil® ODS-3, GL Sciences Inc. Tokyo, Japan) with 5 μm, 4x20 mm guard columns (GL Sciences Inc. Tokyo, Japan). The mobile phase was 50 mmol L^{-1} sodium acetate / 10 % methanol (v/v) at pH 4.2. It was filtered through a 0.2 μm PVDF membrane and degassed in an ultrasonic bath (Wise Clean, WUC-AO3 H, Korea). The flow rate was 0.8 mL min^{-1} and PDA detection was performed at 278 nm (11, 13, 14). The retention times of 3-NT and TYR standards were 35.560 and 10.364 min, respectively. The average percentage of the recoveries of 3-NT and TYR were estimated at 92 % and 90 %, respectively. The limits of detection were 6.7 nmol L^{-1} for 3-NT and 35 nmol L^{-1} for TYR.

Statistical analysis

Means ± standard deviations (SD) of the measured parameters were analysed using the SPSS version 20 (IBM corp., Armonk, NY, USA). Differences between the groups were determined using the analysis of variance (ANOVA), followed by post-hoc testing (Tukey's honestly significant difference - Tukey HSD), and the level for statistical significance was set at *p*<0.01.

RESULTS AND DISCUSSION

Table 1 shows mean 3-NT/1000 TYR ratios in the brain regions for each group. The 3-NT/1000 TYR ratio increased significantly (*p*<0.01) in group 1 and 2 compared to control. In the forebrain, this increase was also significant between the treated groups. However, the within-group differences between brain regions were not significant.

Our study confirms increased brain nitration markers from studies with other OP pesticides such as malathion (15) and chlorpyrifos (16). In contrast, Kose et al. (17) concluded that acute dichlorvos administration did not cause marked oxidative stress in rat heart and that oxidative stress probably did not play a major role in dichlorvos-induced poisoning. However, our study suggests that dichlorvos may cause oxidative stress-mediated neurotoxicity.

Table 1 *Mean±SD values of 3-NT/1000 TYR in different brain regions following dichlorvos treatment*

Groups	Forebrain	Midbrain	Hindbrain
Control	0.053±0.019[a],*	0.056±0.028[a],*	0.047±0.020[a],*
Dichlorvos 1.8 mg kg^{-1}	0.480±0.255[b],*	0.533±0.348[b],*	0.466±0.246[b],*
Dichlorvos 9.0 mg kg^{-1}	0.972±0.168[c],*	0.580±0.365[b],*	0.655±0.369[b],*

Each group comprised 8 rats
[a, b, c] *- values with different superscripts in the same column are significantly different (*p<0.01*)*
** - values are not significantly different between the rows (*p>0.05*).*

Neuromediator and enzyme levels vary in different brain regions. For example, AChE levels, which mediate OP neurotoxicity in the cholinergic pathways, vary in different areas of the brain. High free radical levels and deficient antioxidant defence mechanisms make specific brain regions more susceptible to OP neurotoxicity (18-20). Although the 3-NT level in our study increased in the fore, mid- and hindbrain relative to dose, statistically that increase was not significant between the treated groups in the mid- and hindbrain regions. This suggests that this may be due to different levels of enzyme, neuromediator, and antioxidant defence mechanisms in different parts of the brain.

Even though our study is limited to single-dose-induced nitrative stress in the brain, it has confirmed that 3-NT may play a role in the mechanism of dichlorvos neurotoxicity. Further studies with different doses and different experimental designs, such as chronic toxicity and/or comparisons between dichlorvos and other OPs could help to elucidate the mechanisms which mediate dichlorvos neurotoxicity.

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Sažetak

Razine 3-nitrotirozina kod neurotoksičnosti izazvane diklorvosom

Cilj je ovog ispitivanja bio ocijeniti neurotoksičnost diklorvosa kroz nitrooksidativni stres mjerenjem razina 3-nitrotirozina (3-NT) u prednjem, središnjem i stražnjem režnju mozga akutno izloženih mužjaka štakora Sprague-Dawley, koji su u tu svrhu nasumce bili podijeljeni u tri skupine po osam životinja. Skupina 1 primila je jednokratnu intraperitonealnu dozu diklorvosa od 1,8 mg kg^{-1} (0,1xLD$_{50}$), a skupina 2 dozu od 9 mg kg^{-1} (0,5xLD$_{50}$). Kontrolna je skupina primila 0,5 mL fiziološke otopine, također intraperitonealno. Razine 3-NT-a i tirozina (TIR) izmjerene su tekućinskim kromatografom visoke djelotvornosti s detektorom s nizom dioda (HPLC-PDA) te su izražene kao omjer 3-NT:TIR. Omjeri 3-NT/1000 TIR značajno su se povećali u svim režnjevima izloženih skupina (1 i 2) u odnosu na kontrolnu skupinu (*p*<0,01). Povećanje je također bilo značajno u prednjem režnju skupine 2 u odnosu na skupinu 1, ali nije bilo značajne razlike između izloženih skupina u ostalim režnjevima. Naše je istraživanje potvrdilo da akutna izloženost diklorvosu dovodi do nitrooksidativnog stresa u mozgu te da 3-NT sudjeluje u mehanizmu neurotoksičnosti diklorvosa.

KLJUČNE RIJEČI: *HPLC; mozak; nitrooksidativni stres; pesticidi; štakori*

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