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Oxidative Stress and Free Radical Damage in Patients With Acute Dipterex Poisoning

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Objective To investigate whether acute dipterex poisoning (ADP) may cause oxidative stress and free radical damage in the bodies of acute dipterex poisoning patients (ADPPs), and to explore the mechanisms by which ADP may cause oxidative stress and free radical damage. **Methods** Fifty ADPPs and fifty healthy adult volunteers (HAVs) whose ages, gender and others were matched with the ADPPs were enrolled in a randomized controlled study, in which concentrations of nitric oxide (NO), vitamin C (VC), vitamin E (VE) and β-carotene (β-CAR) in plasma as well as concentration of lipoperoxide (LPO), and activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX) and acetylcholinesterase (AChE) in erythrocytes were determined by spectrophotometric analytical methods. **Results** Compared with the average values of experimental parameters in the HAVs group, the average values of plasma NO and erythrocyte LPO in the ADPPs group were significantly increased (P<0.0001), while those of plasma VC, VE and β-CAR as well as erythrocyte SOD, CAT, GPX and AChE in the ADPPs group were significantly decreased (P<0.0001). Bivariate correlation analysis and partial correlation analysis suggested that when NO and LPO values were increased, and VC, VE, β-CAR, SOD, CAT and GPX values were decreased in the ADPPs, AChE value was decreased gradually in the ADPPs (P<0.001-0.0001). Reliability analysis of experimental parameters reflecting oxidative stress and free radical damage in the ADPPs showed that the reliability coefficient (8 items) alpha=0.6909, and the standardized item alpha=0.8574. **Conclusion** The findings in the present study suggest that ADP can cause oxidative stress and free radical damage, and inhibit markedly erythrocyte acetylcholinesterase activity in ADPPs.

Key words: Dipterex; Dipterex poisoning; Oxidative stress; Free radical damage; Free radicals; Oxidation; Lipoperoxidation; Acetylcholinesterase

INTRODUCTION

Dipterex (dimethyl-2,2,2-trichloro-1-hydroxyethylphosphonate) is one of the organic phosphorous pesticides (OPs) used widely both in rural and urban areas of China. OPs poisoning includes occupational poisoning in industrial production and agricultural application, and suicide, homicide, accidental overdose, accidental overuse and so on[1-3]. Some authors had reported that reactive oxygen species (ROS) reactions, oxidative stress

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and a series of free radical reactions were aggravated markedly in acute OPs poisoning patients, and that levels of vitamin C (VC), vitamin E (VE), β-carotene (β-CAR), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX) and acetylcholinesterase (AChE) were decreased significantly, and those of nitric oxide (NO) and lipoperoxide (LPO) were increased significantly in the patients[3-9]. However, up to now, there have been neither reports on abnormal metabolism or change of the above biochemical substances and enzymes in acute dipterex poisoning patients (ADPPs), nor reports about relationship between oxidative stress, free radical damage and acute dipterex poisoning (ADP). The present study was to investigate whether ADP might cause oxidative stress and free radical damage in the ADPPs, and to explore mechanisms by which ADP might cause oxidative stress and free radical damage to the ADPPs, by means of measuring values of plasma NO, VC, VE and β-CAR as well as those of erythrocyte LPO, SOD, CAT, GPX and AChE in 50 ADPPs and 50 healthy adult volunteers (HAVs), and by means of statistic analyses between experimental parameters in ADPPs and the HAVs.

MATERIALS AND METHODS

Study Design

A randomized controlled design was used in this study. In order to obtain an objective research conclusion, principles of random sampling, control, replication and equilibrium, and management factors, experimental effects and subjects, and inclusion and exclusion criteria of subjects, *etc*. were taken into full consideration, and strictly executed in the study [10-14].

Subjects

Acute dipterex poisoning patients (ADPPs) Fifty ADPPs were randomly sampled from 97 ADPPs in some mountainous areas, villages and towns in the suburbs of Hangzhou, Zhejiang Province, China, by means of "Select Cases-Random Sample of Cases" in "SPSS 11.0 for Windows". The patients were treated in the Second Affiliated Hospital, College of Medicine, Zhejiang University, and their diagnoses were confirmed by history of exposure to dipterex, clinical symptoms and markedly decreased erythrocyte AChE activity. They were all volunteers in this study.

Healthy adult volunteers (HAVs) Fifty HAVs whose age, gender, hemoglobin, serum albumin, body-mass index, annual earning, education level, profession or occupation, and residence region were matched with the ADPPs were randomly sampled from 89 HAVs in the same mountainous areas, villages and towns in the suburbs of Hangzhou, Zhejiang Provience, confirmed by comprehensive physical examination in the Second Affiliated Hospital, College of Medicine, Zhejiang University, by means of "Select Cases-Random Sample of Cases" in "SPSS 11.0 for Windows". They were all volunteers in this study.

There was no significant difference in the average values of age, hemoglobin, serum albumin and body-mass index between the ADPPs group and the HAVs group. There was neither significant difference in the gender, annual earning, education level, profession or occupation, and residence region (mountainous areas, villages and countries) between the two groups.

The demographic data and some other data of 50 ADPPs and 50 HAVs are presented in Table 1.

The ADPPs and HAVs with a medical history of disorders associated with brain, heart, lung, liver, kidney and other organs as well as blood, circulatory, respiratory, digestive and

other systems were all excluded in this study. Those with a medical history of inflammation, hypertension, hyperlipidemia, acute or chronic bronchitis, autoimmune disease, diabetes, atherosclerosis, tumors and other diseases as well as subnutrition, malnutrition, supernutrition and other nutritional diseases were also all excluded in this study. In addition, the above subjects had no smoking and excessive drinking history.

TABLE 1

Demographic Data and Some Other Data in ADPPs and HAVs Groups

Items	ADPPs (n=50)	HAVs (n=50)	Statistic Analysis
Age (year)	18-38	20-38	t=0.4344[a]
	(23.8±5.0)	(24.2±4.6)	P=0.6650
Gender	M=21	M=25	χ^2=0.6441[b]
	F=29	F=25	P=0.5475
Hemoglobin (g/L)	115-138	113-141	t=0.7651[a]
	(129.06±5.54)	(129.97±6.39)	P=0.4461
Albumin (g/L)	36.44-47.56	36.33-43.11	t=1.5891[a]
	(40.96±2.16)	(40.34±1.73)	P=0.1153
Body-mass Index	18.43-24.12	18.39-24.35	t=0.1119[a]
	(22.55±1.11)	(22.30±1.13)	P=0.2689
Smoking History	No	No	—
Abusing Alcohol History	No	No	—

Note. [a]Independent samples t test; [b]Pearson Chi-square test (exact sig.).

The subjects were exposed neither to radiation, nor to intoxicating materials or pesticides. Within the prior month before they volunteered for this study, none of them had taken any antioxidant supplements such as VC, VE, β-CAR, ginkgo biloba, tea polyphenols or other similar substances.

Methods

Collection and pretreatment of blood samples Venous blood samples were collected from the ADPPs at the time of providing emergency care, and fasting venous blood samples were collected from the HAVs in the morning, and heparin sodium was added as anticoagulant. The plasma and erythrocytes separated promptly were stored at -50℃ immediately. The blood samples collected did not undergo any hemolysis[10,11,15,16].

Determination of biochemical substances and enzymes The spectrophotometric analytical method (SAM) of α-naphthylamine coloration was used to determine plasma NO level expressed as nmol/L[15,16]. The SAM of thiobarbituric acid reactive substances (TBARS) was used to determine erythrocyte LPO level expressed as nmol/g·Hb[10,11,15,16]. The SAM of ferrozine coloration was used to determine plasma VC and VE levels expressed as μmol/L[10,11,15,16]. The plasma β-CAR level extracted with a mixture of ethanol and petroleum ether was determined by SAM and expressed as μmol/L[10,11,15,16]. The SAM of inhibiting pyrogallol auto-oxidation was used to determine erythrocyte SOD activity expressed as U/g·Hb[10,11,15,16]. The SAM of coloration of hydrogen peroxide and acetic acid-potassium dichromate was used to determine erythrocyte CAT activity expressed as K/g·Hb[10,11,15,16]. The modified Hafeman's SAM was used to determine erythrocyte GPX

activity expressed as U/mg · Hb[15,16].

Determination of erythrocyte AChE activity 0.05 mL hemolytic solution in which white blood cells and platelets were removed and the hemoglobin concentration was measured, 0.95 mL phosphate buffer (0.067 mol/L, pH 7.20) and 1.0 mL acetylcholine chloride solution (10.0 μmol/L) prepared by acetate buffer (1.0 mmol/L, pH 4.50) were mixed. After 20 min in a water bath at 37℃, 4.0 mL alkalescent hydroxylamine hydrochloride solution mixed by hydroxylamine hydrochloride solution (140.0 g/L) and sodium hydroxide solution (3.50 mol/L) = 1:1, 2.0 mL hydrochloric acid (ρ_{20}=1.19 g/mL) and 2.0 mL ferric chloride solution (0.60 mol/L) was added. After centrifugation at 3 000 rpm for 5 min, the supernatant liquid was used to determine the AChE activity with the spectrophotometric analytical method at the wavelength of 530 nm (1.0 cm cell). Acetylcholine chloride solution (10.0 μmol/L) was used as the standard, and the erythrocyte AChE activity was expressed as U/g · Hb[3,17]. In the methodology of determining erythrocyte AChE activity, the coefficient of variation (CV) was 2.08% (249.75±5.19, n=20), the recovery rate ranged from 97.35% to 102.67% (100.59%±1.48%, n=20), the colorating linearity at 25℃ was r=0.9984, P<0.0001 (n=23), and the coloration stability at 25℃ ranged from 5 minutes to 12 hours (n=20).

In determining the above biochemical substances and enzymes, the main analytical reagents, such as vitamin C, vitamin E, β-carotene, Cu · Zn-superoxide dismutase, catalase, 5,6-diphenyl-3-(2-pyridyl)-1,2,4-triazinedisulfonic acid disodium salt (ferrozine), 1,2,3-trihydroxybenzene (pyrogallol), 1,1,3,3-tetraethoxypropane, 2-thiobarbituric acid (TBA), α-naphthylamine, acetylcholine chloride, *etc*. were purchased from SIGMA® Chemical Company, USA. The other analytical reagents were produced in China. The fresh quadruply distilled water was prepared with a quartz glass distilling apparatus. In the determination of the above biochemical substances and enzymes, the main analytical instruments included Hewlett Packard 8453-Spectrophotometer, USA, and others.

In the determination of the above biochemical substances and enzymes, the standardization during experimenting processes, such as the same batch number of each reagent, the same quality control, the same laboratory assistant, and the identical analytical apparatus, was strictly used for each experiment in order to decrease errors and biases, and to ensure the analytical quality of the tests[10-14].

Medical Statistic Analysis

All data in this study were statistically analyzed with SPSS 11.0 for Windows statistic software using a Compaq Pentium Ⅳ/2.4 GHz computer. In this study the biochemical parameters presented all normal distributions by Kolmogorov-Smirnov Z test and were expressed as $\bar{x}\pm s$ and 95% confidence interval (95% CI). Hypothesis testing methods included independent-sample t test, Pearson Chi-square test (χ^2 test), etc. At the same time, bivariate correlation was used to analyze the relationships between erythrocyte AChE activity and other biochemical parameters. After age factor was eliminated or controlled the partial correlation analysis was used to analyze the relationships between AChE and other biochemical parameters, and reliability analysis was used to prove dependability of the experiment parameters reflecting oxidative stress and others. In the statistical analysis in this study, the level of hypothesis testing (α) was ≤0.05 in order to avoid false positives (Type Ⅰ error), and the power of hypothesis testing (power) was ≥0.85 in order to avoid false negatives (Type Ⅱ error)[10-14].

TABLE 2

Comparison of Average Values ($\bar{x} \pm s$) of Biochemical Parameters Between ADPPs and HAVs Groups and Their 95% CI

Group	*n*	Plasma				Erythrocytes				
		NO	VC	VE	β-CAR	LPO	SOD	CAT	GPX	AChE
		nmol/L	μmol/L	μmol/L	μmol/L	nmol/g • Hb	U/g • Hb	K/g • Hb	U/mg • Hb	U/g • Hb
ADPPs	50	549.6±135.8 (511.0-588.2)	37.82±10.43 (34.85-40.78)	16.79±4.77 (15.43-18.14)	1.21±0.36 (1.11-1.32)	35.27±5.58 (33.68-36.86)	1790±161 (1744-1836)	230.6±69.2 (211.0-250.3)	20.49±5.76 (18.85-22.13)	213.8±60.0 (196.8-230.8)
HAVs	50	359.7±87.8 (334.7-384.6)	56.32±16.74 (51.56-61.08)	26.08±7.60 (23.92-28.24)	1.73±0.53 (1.58-1.88)	28.15±4.84 (26.78-29.53)	2135±193 (2080-2190)	291.5±87.4 (266.7-316.4)	28.84±8.16 (26.52-31.16)	308.0±86.4 (283.5-332.6)
t[a]		8.3043	6.6329	7.3286	5.7171	6.8161	9.7088	3.8641	5.9125	6.3377
P		< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.0002	< 0.0001	< 0.0001

Note. [a]Independent-samples *t* test. Figures in parentheses are 95% CI.

RESULTS

Comparison of Average Values of Biochemical Parameters ($\bar{x} \pm s$) Between ADPPs Group and HAVs Group

Compared with the average values of biochemical parameters in the HAVs group, the average values of plasma NO and erythrocyte LPO in the ADPPs group were significantly increased ($P<0.0001$), while the average values of plasma VC, VE and β-CAR as well as those of erythrocyte SOD, CAT, GPX and AChE in the ADPPs group were significantly decreased ($P<0.0001$) (Table 2).

95% Confidence Interval of Average Values of Biochemical Parameters ($\bar{x} \pm s$) in ADPPs and HAVs Groups

The lower limits of 95% confidence interval (95% CI) of the average values of NO and LPO in the ADPPs group were greater than the upper limits of 95% CI of those in the HAVs group. The upper limits of 95% CI of the average values of VC, VE, β-CAR, SOD, CAT, GPX and AChE in the ADPPs group were less than the lower limits of 95% CI of those in the HAVs group (Table 2).

Bivariate Correlation Analysis of Erythrocyte AChE Value and Each of Other Biochemical Parameters in 50 ADPPs

The bivariate correlation was used to analyze whether there existed correlations between erythrocyte AChE value and each of other biochemical parameters in the ADPPs group. The findings showed that when NO and LPO were increased and VC, VE, β-CAR, SOD, CAT and GPX were decreased in the ADPPs, AchE was decreased gradually ($P<0.0001$) (Table 3).

TABLE 3

Bivariate Correlation Analysis Between Erythrocyte AChE Value and Each of Other Biochemical Parameters in ADPPs

Correlative Items	*n*	*r*	*t*	*P*
AChE With NO	50	−0.6146	5.3983	< 0.0001
AChE With VC	50	0.6866	6.5420	< 0.0001
AChE With VE	50	0.6211	5.4887	< 0.0001
AChE With β-CAR	50	0.6928	6.6730	< 0.0001
AChE With LPO	50	−0.7809	8.6623	< 0.0001
AChE With SOD	50	0.7448	7.7376	< 0.0001
AChE With CAT	50	0.6586	6.0624	< 0.0001
AChE With GPX	50	0.8572	11.5367	< 0.0001

Partial Correlation Analysis of Erythrocyte AChE Value and Each of Other Biochemical Parameters in 50 ADPPs

Owing to a close correlation between age and each of the above biochemical parameters in the ADPPs and HAVs groups[3,10,11,15-20,23-26,48-53], the partial correlation was analyzed in

order to eliminate the effect of age upon the above biochemical parameters in bivariate correlation analysis, and to show the dependability of the bivariate correlation. The findings further suggested that NO and LPO were increased and VC, VE, β-CAR, SOD, CAT and GPX were decreased in the ADPPs, and AChE was decreased gradually (P<0.001-0.0001) (Table 4).

TABLE 4

Partial Correlation Analysis Between Erythrocyte AChE Value and Each of Other Biochemical Parameters in ADPPs

Correlative Items	*n*	Controlling for	*r*	*P*
AChE With NO	50	Age	-0.5688	< 0.001
AChE With VC	50	Age	0.5467	< 0.001
AChE With VE	50	Age	0.4435	0.001
AChE With β-CAR	50	Age	0.5498	< 0.001
AChE With LPO	50	Age	-0.7309	< 0.0001
AChE With SOD	50	Age	0.6202	< 0.0001
AChE With CAT	50	Age	0.5227	< 0.001
AChE With GPX	50	Age	0.8094	< 0.0001

Reliability Analysis of Biochemical Parameters Reflecting Oxidative Stress and Free Radical Damage to ADPPs

The findings suggested that the reliability coefficient (8 items) alpha=0.6909, P<0.0001, and the standardized item alpha=0.8574, P<0.0001.

DISCUSSION

VC, VE and β-CAR are important antioxidants, SOD, CAT and GPX are important antioxidases, and LPO is a product of peroxidative reactions (auto-oxidation) of lipids exposed to oxygen. They all play very important roles in healthy status of the human[3,9-11,15-26]. NO is one of the very important neurotransmitter molecules, and can directly modify enzymes that produce second messengers, so it plays an important role in the metabolism of the human[15,16,18,19,21-25,26-28]. Markedly increased NO and LPO as well as significantly decreased antioxidants and antioxidases may induce metabolic disorders and pathological aggravation of a series of free radical chain reactions, thus resulting in oxidative stress and a variety of diseases associated with abnormal reactions of free radicals (FRs) in the human[3,9-11,15-33].

The findings in this study showed that there existed an abnormal metabolism of NO as well as an imbalance between oxidation and antioxidation, and an oxidative stress and free radical damage in the ADPPs. There might be several interpretations.

Dipterex in alimentary tract and stomach is combined rapidly with hydrochloric acid and other organic acids to produce a series of vehement chemical and biochemical reactions (CBRs), thus producing a large amount of deadly toxins[2,3,5,6,10,18,33-37]. At the same time, CBRs produce and release a large number of FRs such as superoxide anion radical ($O_2^{\cdot-}$), hydroxyl radical (·OH) and others as well as ROS like singlet oxygen ($^1O_2$), hydrogen

peroxide (H_2O_2), thus promoting the violent aggravation of a series of free radical chain reactions, attacking mucous membranes of the alimentary tract and many important organs and tissues, producing poisoning and inflammatory CBRs[2,3,5,6,10,18,33-37]. Subsequently, dipterex and its metabolites, and the deadly toxins produced by CBRs are absorbed largely by mucous membranes in the alimentary tract, which present a series of metabolic disturbances, resulting in toxic symptoms such as extensive bleeding in intestines and other organs and tissues[2,3,5,6,10,18,33-37]. In ADPPs cytokines released by inflammatory reactions, especially interleukin-1, and the abnormal metabolism of cytochromes P-450 induced by CBRs as well as the aggravation of xanthine/xanthine oxidase reactions induced by bleeding and other toxic symptoms, could produce and release a large amount of FRs and ROS, thereby deactivating antioxidants and antioxidases, catalyzing and aggravating lipoperoxidative reactions of polyunsaturated fatty acids (PUFAs) and other lipids, damaging cellular functions[2,3,5,6,10,18,26,33-38]. As a result, the level of erythrocyte LPO would be significantly increased, and the levels of plasma VC, VE and β-CAR as well as the activities of erythrocyte SOD, CAT and GPX would be significantly decreased in ADPPs. In addition, cytokines, inflammatory reactions and CBRs in the ADPPs might activate inducible nitric oxide synthase (INOS), thus catalyzing and producing a large amount of NO[3,6,9,23-25].

Serum or plasma cholinesterase (BChE, EC 3.1.1.8)[36] and erythrocyte acetylcholinesterase (AChE, EC 3.1.1.7)[36] activities play an important role in monitoring persons at risk of dipterex and other OPs exposure and in diagnosing patients with dipterex and other OPs poisoning[1-3,36-47]. However, the determination of erythrocyte AChE activity is a more appropriate tool for the diagnosis of dipterex and other OPs exposure and poisoning, because AChE in serum or plasma, liver, heart, pancreas and brain tissues is *per se* a pseudocholinesterase, namely, a false cholinesterase, whereas erythrocyte AChE is a true cholinesterase[2,3,17,43,45]. In addition, erythrocyte AChE activity is better than serum or plasma cholinesterase activity in reflecting dipterex and other OPs poisoning degrees, as serum or plasma cholinesterase activity is markedly disturbed by the changes of serum or plasma volume and other factors[2,3,17,43,45]. In this study, erythrocyte AChE activity in the ADPPs was significantly inhibited. The reason was that the active sites and groups in AChE structures were attacked and destroyed by the covalent binding of phosphate FRs in dipterex, and other excessive FRs and ROS. As a result, AChE was transformed into enzymatically inert proteins due to the AChE-dipterex interaction process[2,3,17,41].

The findings of the bivariate correlation analysis in erythrocyte AChE activity and other biochemical parameters in this study suggested that when NO and LPO were increased and VC, VE, β-CAR, SOD, CAT and GPX were decreased in the ADPPs, AChE was decreased gradually. At the same time, the findings also suggested that the lower the erythrocyte AChE activity, the severer the oxidative stress and the free radical damage in the ADPPs. In other words, the deeper the ADP poisoning, the more grievous the oxidative stress and the free radical damage in the ADPPs[2,3,17].

The findings of the partial correlation analysis after age factor was eliminated or controlled in this study, suggested that when NO and LPO were increased and VC, VE, β-CAR, SOD, CAT and GPX were decreased in the ADPPs, AChE was decreased gradually. Additionally, the findings objectively reflected the relationships between AChE and other biochemical parameters, and between AChE and ADP poisoning degrees in patients[2,3,17].

In this study the reliability analysis was computed in order to estimate the dependability of the experimental biochemical parameters that reflected the oxidative stress and free

radical damage to the ADPPs[12-14]. The findings showed that the experimental parameters were, to a large extent, comparatively satisfactory and reliable (the reliability coefficient of 8 items: alpha=0.6829, $P<0.0001$, and the standardized item alpha=0.8522, $P<0.0001$). Regretfully, limitations existed in this study. For instance, the TBARS method was used to determine erythrocyte LPO level. Although erythrocyte LPO level reflecting the lipoperoxidative reactions of PUFAs and other lipids is more appropriate than serum or plasma LPO level[10,11], it is not very accurate, thereby reducing the reliability of the experimental parameters.

In conclusion, the findings of the present study suggest that acute dipterex poisoning can cause the aggravation of a series of free radical chain reactions and oxidative stress in acute dipterex poisoning patients, thereby leading to free radical damage, and can inhibit markedly erythrocyte acetylcholinesterase activity.

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