Beneficial Effect of N-Acetylcysteine against Organophosphate Toxicity in Mice

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Organophosphate (OP) compounds are the most common chemical agents that cause pesticide poisoning. Presently, more than 100 different OP compounds are used worldwide as insecticides.1,2 Because of widespread use and easy accessibility, these compounds result in a huge number of intoxications. Between 750000 and 3000000 human intoxications per year are estimated worldwide,1,2 resulting in several hundred thousands of fatalities annually.3 Accidental exposure is the main cause for mild poisonings23,4 and severe cases are mostly due to suicidal use.5—7

Most of OP compounds are highly lipid-soluble agents and are well absorbed from the skin, oral mucous membranes, conjunctiva and gastrointestinal and respiratory tracts. The onset, severity and duration of poisoning is determined by the dose, route of exposure, physicochemical properties of the OP (e.g. lipid solubility), rate of metabolism (whether transformation in the liver is required before the compound becomes toxic) and whether the organophosphorylated cholinesterase ages rapidly.8

Toxicity from OP compounds is primarily through the inhibition of acetylcholinesterase (AChE) activity.9 However, recent studies showed that oxidative stress could be an important component of the mechanism of OP compounds toxicity. OP compounds may induce oxidative stress leading to generation of free radicals and alterations in antioxidants status or reactive oxygen species (ROS) scavenging enzymes.10—12 Therefore, OP compounds may enhance lipid peroxidation (LPO) by directly interacting with the cellular membrane and ROS.13 Some studies showed that LPO has been suggested as one of the molecular mechanisms involved in OP compounds induced toxicity.10,14 Therefore, treatment with antioxidants and free radical scavengers can decrease the oxidative stress related to OP-induced toxicity.

N-Acetylcysteine (NAC) is a thiol compound with potent antioxidant and antiinflammatory properties.15 Its antioxidant action is believed to originate from its ability to stimulate reduced glutathione (GSH) synthesis, therefore maintaining intracellular GSH levels and scavenging ROS.16 NAC is used clinically to treat several diseases related to oxidative stress and/or GSH deficiency such as paracetamol (acetaminophen) overdose, VIH infection, and lung and heart diseases.17—18 It has also been proven to be useful in the treatment of acute paraquat and heavy metal poisoning.19—21 Another study demonstrated that NAC may be a complementary antioxidant for OP poisoning, acting at different target sites.22 All of these beneficial properties led us to consider NAC as a strong candidate for restoring impaired prooxidant/antioxidant balance in OP poisoning.23

The aim of present study was to investigate prophylactic and therapeutic effects of NAC against fenthion-induced oxidative stress in mice. Additionally, the effects on survival rates were investigated. Therefore, we determined the changes of the blood levels of glutathione (GSH), malondialdehyde (MDA), nitrite, and nitrate in blood or serum. Additionally, all animals were observed for 6 h and the survival rates were recorded. It was found that fenthion administration increased the levels of MDA, and decreased the levels of GSH, nitrite and nitrate. On the other hand, both prophylactic and therapeutic NAC treatment decreased the levels of MDA, and increased the levels of GSH, nitrite, and nitrate. The results showed that NAC is able to attenuate the fenthion-induced oxidative stress whereby NAC has not only prophylactic but also therapeutic activity in fenthion poisoning. On the other hand, we found that NAC can clearly improve survival rates in mice administered with an acute high dose of fenthion poisoning. In conclusion, NAC can decrease OP-induced oxidative stress and mortality rate, but the exact mechanism of its NAC protective effect needs to be explored further.

Key words  N-acetylcysteine; fenthion; mice; oxidative stress; survival rate

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Materials and Methods

Chemicals Fenthion was obtained from Bayer Crop Science (East Hawthorn, Australia). NAC was purchased from Hüsnü Arsan Co. (Istanbul, Turkey), GSH, thiobarbituric acid, phosphate buffer, butylated hydroxytoluene, trichloroacetic acid, ethylenediaminetetraacetic acid, [5,5-dithiobis-(2-nitrobenzoic acid)], phenylendiamine, sodium nitrite, sodium nitrate, sulfanilamide, N-(1-naphthyl)ethylenediamine dihydrochloride and vanadium(III) chloride were purchased from Sigma Chemical Co. All other chemicals and

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reagents used in this study were analytical grade. Fenthion and NAC were diluted in saline.

**Animals** Male Swiss mice weighing 30—35 g were housed under standard laboratory conditions and were allowed free access to food and water. These experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Afyon Kocatepe and were in accordance with National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publication, vol. 25, no. 28, 1996).

**Experimental Design** Thirty mice were divided into four groups. Group 1 (sham, n=6) did not receive any agent, and groups 2, 3, and 4 received subcutaneous fenthion (0.1 g/kg). Group 2 (fenthion+saline, n=8) was also given intraperitoneally (i.p.) normal saline (same volume as NAC). Group 3 (fenthion+prophylactic NAC, n=8) received 150 mg/kg of NAC (i.p.) 1 h before fenthion injection. Group 4 (fenthion+therapeutic NAC, n=8) received 150 mg/kg of NAC (i.p.) 1 h after fenthion injection. Twenty four hours later, all mice were anesthetized with ketamine hydrochloride (50 mg/kg, i.p.), and blood samples were taken by cardiac puncture for biochemical analysis. Then, the animals were sacrificed under anesthesia.

**Biochemical Analysis** Fasting blood samples were drawn into heparin-free tubes during routine blood sampling for biochemical analyses. After immediate centrifugation (1000 g for 10 min at +4 °C), the serum was stored in a polystyrene plastic tube at −70 °C until the time of analysis. Whole blood was collected into heparinized tubes and whole blood MDA and GSH levels were studied on the same day of admission. Whole blood MDA (as an important indicator of oxidative stress) levels were measured according to a method of Jain et al.23) The principle of the method is based on the spectrophotometric measurement of the color that occurred during the reaction of thiobarbituric acid with MDA. Concentrations of thiobarbituric acid reactive substances (TBARS) were calculated by the absorbance coefficient of malondialdehyde-thiobarbituric acid complex and expressed in nmol/ml. Whole blood GSH concentration was also measured by a spectrophotometric method.24) The concentrations of nitric oxide (nitrite and nitrate) were detected by the methods of Miranda et al.25) Nitrite and nitrate calibration standards were prepared by diluting sodium nitrite and sodium nitrate in pure water. After loading the plate with samples (100 µl), addition of vanadium(III) chloride (100 µl) to each well was rapidly followed by addition of the Griess reagents, sulfanilamide (50 µl) and N-(1-naphthyl)ethylenediamine dihydrochloride (50 µl). The Griess solutions may also be pre-mixed immediately prior to application to the plate. Nitrite mixed with Griess reagents forms a chromophore from the diazotization of sulfanilamide by acidic nitrite followed by coupling with bicyclic amines, such as N-1-(naphthyl)ethylenediamine. Sample blank values were obtained by substituting diluting medium for Griess reagent. Nitrite was measured in a similar manner except that samples and nitrite standards were only exposed to Griess reagents. The absorbance at 540 nm was read to assess the total level of nitrite and nitrate in all samples.25)

**Mortality Study** Forty mice were randomly divided into five equal groups (n=8). Group A (control; fenthion+normal saline) received 0.2 g/kg fenthion i.p. followed by i.p. normal saline 1 h later. Group B (fenthion+atropine) received 0.2 g/kg fenthion i.p. followed by i.p. 5 mg/kg Atropine 1 h later. The other 3 groups (C, D, and E; fenthion+NAC) received 0.2 g/kg fenthion i.p. followed by NAC 50, 100, and 200 mg/kg (i.p.) 1 h later, respectively. Normal saline, Atropine, and NAC were administered in equal volume. Mortality (cessation of movement, including respiration) was assessed by two investigators and recorded at 1, 3, and 6 h. All of the animals were sacrificed by cervical dislocation.

**Statistical Analysis** Data in biochemical analysis were expressed as mean±standard deviation (S.D.). Statistical analysis of data obtained in the oxidative stress were performed using a one-way analysis of variance (ANOVA) and Tukey’s posttest. A value of p<0.05 was considered statistically significant. The Kaplan–Meier method was applied to delineate the survival curves of the results in mortality study. The difference between the two survival curves was estimated by the logrank test.

**RESULTS**

**MDA Levels** We determined that blood MDA levels were minimal in Group 1 (1.17±0.1 nmol/ml) and maximal in Group 2 (1.91±0.5 nmol/ml) in Group 2 (1.24±0.3 nmol/ml) and therapeutic (Group 4, 1.25±0.1 nmol/ml) treatment of NAC significantly decreased the levels of MDA compared with group 2 (p<0.001). Additionally, there was no difference between Group 3 and 4 blood MDA levels (Fig. 1).

**Glutathione Levels** In Group 2 blood GSH levels (29.87±3.3 mg/dl) were significantly lower than that in Group 1 (24.36±3.1 mg/dl, p<0.05). Besides, blood GSH levels in Group 2 were significantly lower than those in mice in Groups 3 (28.87±2.9 mg/dl, p<0.05) and 4 (31.49±2.7 mg/dl, p<0.01). There was no difference between Group 3 and 4 blood GSH levels (Fig. 2).

**Nitrite Levels** Serum nitrite levels were minimal in Group 2 (1.30±0.4 mg/dl) and maximal in Group 4 (2.85±0.7 mg/dl). Serum nitrite levels in Group 2 were insignificantly lower than those in Group 1 (1.59±0.2 mg/dl). On the other hand, nitrite levels significantly increased in Groups 3 (2.16±0.3 mg/dl) and 4 (2.85±0.7) with respect to Group 2. Serum nitrite levels in Group 4 were higher than that of

![Fig. 1. Effect of N-Acetylcysteine (NAC) on Blood Levels of Malondialdehyde (MDA) in Mice 24 h after Fenthion Administration](image-url)
Group 3 (Fig. 3).

**Nitrate Levels** The minimal serum nitrate levels were seen in Group 2 (5.11 ± 1.3 mg/dl) but the maximal serum nitrate levels were present in Group 4 (7.85 ± 1.5 mg/dl). Serum nitrate levels in Group 2 were insignificantly lower than those in Group 1 (5.84 ± 1.6 mg/dl). Serum nitrate levels in Group 3 (6.57 ± 0.9 mg/dl) were higher than that of Group 2, but there was no significant difference. Additionally, nitrate levels in Group 4 were higher than those in Group 3 (Fig. 4).

**Mortality Study** The highest mortality rate was observed in group A during the first hour and survival rate was 12.5% within 6h. The mortality rates of NAC treated mice (Groups C, D and E) at the first hour were lower than that of Group A. The survival rates for Groups C, D and E were 37.5%, 37.5% and 62.5%, respectively, within 6h. There was no dead mouse in Group B during sixth hours (Fig. 5). But only atropine (Group B) and NAC 200 mg/kg (Group E) effects were statistically significant with respect to Group A.

**DISCUSSION**

The primary effect of fenthion and other OP compounds are the inhibition of the AChE. However, the effects of OP compounds are not restricted to AChE inhibition. It has been reported that OP compounds, besides their inhibitory effect on AChE, also induce oxidative stress.\(^{22,26,27}\) Oxidative stress occurs when the critical balance between oxidants and antioxidants is disrupted due to the depletion of antioxidants or excessive accumulation of the ROS, or both, leading to damage.\(^{27,28}\) ROS such as hydrogen peroxide (H\(_2\)O\(_2\)) and the free radicals superoxide (O\(^2-\)) and hydroxyl radical (HO\(^-\)) can react with biological macromolecules and produce enzyme inactivation, lipid peroxidation, and DNA damage, resulting in oxidative stress. Pesticides are recently known to be able to induce in vitro and in vivo generation of ROS.\(^{10}\) Despite the potential danger of ROS, cells present a variety of defense mechanisms to neutralize the harmful effects of free radicals. The antioxidant defense system includes enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), glutathione S-transferase (GST) and
low molecular weight scavengers such as GSH.\(^{27}\)

LPO has been reported as a major contributor to the loss of cell function under oxidative stress conditions.\(^{23}\) MDA is an indicator of LPO, and its level increases in tissues when they are exposed to oxidative stress.\(^{29}\) It is reported that OP compounds-induced oxidative stress as shown by enhanced MDA production.\(^{29}\) In the present study, we observed that MDA levels increased in fenthion poisoning group, but it returned to control level in prophylactic and therapeutic NAC treatment groups.

GSH is a ubiquitous thiol-containing tripeptide that is involved in numerous processes that are essential for normal biological function, such as DNA and protein synthesis. It is predominantly present in cells in its reduced form (GSH), which is the active state. Among the several important functions of GSH, it contributes to the removal of reactive electrophiles (such as many metabolites formed by the cytochrome P-450 system) through conjugation by means of GSTs. GSH also scavenges ROS directly or in a reaction catalysed by GPx through the oxidation of two molecules of GSH to a molecule of glutathione disulphide (GS(G)S). The relationship between the reduced and oxidised state of GSH, the GSH/GS(G)S ratio or glutathione redox status, is considered as an index of the cellular redox status and a biomarker of oxidative damage, because GSH maintains the thiol-disulphide status of proteins, acting as a redox buffer.\(^{22}\)

Depletion of reduced GSH results in decreased antioxidant capacity of the cell, accumulation of ROS, and ultimately mitochondrial impairments if not compensated otherwise. GSH depletion and oxidation was observed in human lymphocytes, and plasma, in rat blood, liver, and the brain of rats exposed to OP and nerve agents.\(^{31}\) Hai et al.\(^{26}\) found decreased levels of GSH in carp liver and muscle after 24 h exposure to 1 and 5 mg/l of dichlorvos, an OP insecticide known to induce oxidative damages. In human poisoning cases, lindane (organochlorine) and malathion (OP) also decreased blood GSH content.\(^{27}\) Similarly, our results revealed that GSH level decreased during fenthion induced toxicity. NAC, a precursor of glutathione and also a free radical scavenger, has been demonstrated to increase the glutathione levels in vivo and in vitro when administered prior to paraquat exposure.\(^{29}\)

In our study, we treated the mice with NAC 1 h before and after fenthion poisoning, and we determined that both of prophylactic and therapeutic NAC treatments restored the GSH levels. There was no difference between prophylactic and therapeutic effectiveness.

Nitric oxide (NO), as a free radical, seems to be a potential antioxidant. It takes part in termination of LPO reactions. NO is an effective chain-breaking antioxidant in free radical-mediated LPO. It reacts rapidly with peroxyl radicals as a sacrificial chain-terminating antioxidant. The antioxidant effect of NO on LPO has been explained by terminating the radical chain reaction through the reaction of NO with the lipid peroxyl radical (ROO·) to form adducts by below equation.

\[
4\text{NO} + 2\text{ROO}^- + \text{H}_2\text{O} \rightarrow 2\text{RONO} + 2\text{NO} + \text{H}_2\text{O} \rightarrow \text{RONO}_2 + \text{RONO} + 2\text{HNO}_2
\]

A protective effect of NO on LPO has been shown by some investigators.\(^{32,33}\) In the present study, we also found that LPO was increased while the serum levels of nitrate and nitrite were decreased in untreated OP-poisoning mice. Again, both prophylactic and therapeutic NAC treatments increased the nitrate and nitrite levels with compared to control or untreated mice. Effect of NAC on NO pathway, may be mediated either by an activation of NOS or by an inhibition of NO degradation. Acute treatment with NAC was found to potentiate the hypotensive effect of captopril and enalaprilat in spontaneously hypertensive rats via NO-dependent mechanism.\(^{39}\) Moreover, it has also been demonstrated that NAC treatment increased endothelial and/or neuronal nitric oxide synthase activity in spontaneously hypertensive rats.\(^{35,36}\) Based on mentioned above, it may be suggested that the protective effect of NAC against oxidative stress, at least in part, may be related to restoration of NO availability.

OP compounds, used commonly in agriculture, are a gradual increasing cause of accidental and suicidal poisoning, with high morbidity and mortality rates, especially in developing countries.\(^{37}\) For example, about 13000 hospital admissions and 1000 deaths associated with pesticide poisoning occur per year among a Sri Lankan population of less than 15 million, and in China an estimated 100000 cases of pesticide poisoning occur each year.\(^{28}\) In Germany, emergency physicians have to treat about 200 cases annually, mostly due to suicide attempts resulting in 30% lethality.\(^{39}\) Therefore, it appears rational to demonstrate the opportunities, but also the limitations of an optimized therapeutic regimen in OP poisoning.\(^{43}\) At the present, the use of the traditional antidotes, atropine and oximes, has not significantly reduced the morbidity and mortality of OP poisoning, despite the great advances in patient monitoring and critical care. The need to develop newer treatment regimes is urgent.\(^{40}\) Peña-Llopis et al.\(^{22}\) demonstrates that NAC may be a complementary antidote for OP poisoning, acting at different target sites.\(^{25}\) NAC treatment is able to improve paraquat-induced mortality significantly in rats.\(^{41}\) Peña-Llopis et al.\(^{22}\) demonstrated that, NAC increased fish survival following exposure to a lethal dose of the OP pesticide dichlorvos. In our study we found that NAC treatment increased survival rates after fenthion exposure. Especially, survival of the groups treated with 200 mg/kg NAC were higher than NAC 50 and 100 mg/kg treatment groups. Therefore, in the present study thought that the most of suitable dose of NAC is 200 mg/kg i.p. in mice. In addition, NAC characteristics, such as its low toxicity, low price, and high solubility makes it suitable for treatment of OP intoxications.\(^{22}\)

CONCLUSIONS

The present study demonstrates some major findings regarding the effects of NAC on OP toxicity. First, we showed that NAC is able to attenuate the OP-induced oxidative stress in mouse. Second, we revealed that NAC has not only prophylactic but also therapeutic activity in OP-poisoning. Third, we found that NAC can clearly improve survival rates in mice administered with an acute high dose of OP. The exact mechanism of NAC protective effect needs to be explored further.

REFERENCES
