

# Attenuation of phosphamidon-induced oxidative stress and immune dysfunction in rats treated with *N*-acetylcysteine

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The effect of *N*-acetylcysteine, a thiolic antioxidant, on attenuation of phosphamidon-induced oxidative stress and immune dysfunction was evaluated in adult male Wistar rats weighing 200-250 g. Rats were divided into four groups, 8 animals/group, and treated with phosphamidon, *N*-acetylcysteine or the combination of both for 28 days. Oral administration of phosphamidon (1.74 mg/kg), an organophosphate insecticide, increased serum malondialdehyde ( $3.83 \pm 0.18$  vs  $2.91 \pm 0.24$  nmol/mL;  $P < 0.05$ ) and decreased erythrocyte superoxide dismutase ( $567.8 \pm 24.36$  vs  $749.16 \pm 102.61$  U/gHb;  $P < 0.05$ ), catalase activity ( $1.86 \pm 0.18$  vs  $2.43 \pm 0.08$  U/gHb;  $P < 0.05$ ) and whole blood glutathione levels ( $1.25 \pm 0.21$  vs  $2.28 \pm 0.08$  mg/gHb;  $P < 0.05$ ) showing phosphamidon-induced oxidative stress. Phosphamidon exposure markedly suppressed humoral immune response as assessed by antibody titer to ovalbumin ( $4.71 \pm 0.51$  vs  $8.00 \pm 0.12$   $-\log_2$ ;  $P < 0.05$ ), and cell-mediated immune response as assessed by leukocyte migration inhibition ( $25.24 \pm 1.04$  vs  $70.8 \pm 1.09\%$ ;  $P < 0.05$ ) and macrophage migration inhibition ( $20.38 \pm 0.99$  vs  $67.16 \pm 5.30\%$ ;  $P < 0.05$ ) response. Phosphamidon exposure decreased IFN- $\gamma$  levels ( $40.7 \pm 3.21$  vs  $55.84 \pm 3.02$  pg/mL;  $P < 0.05$ ) suggesting a profound effect of phosphamidon on cell-mediated immune response. A phosphamidon-induced increase in TNF- $\alpha$  level ( $64.19 \pm 6.0$  vs  $23.16 \pm 4.0$  pg/mL;  $P < 0.05$ ) suggests a contributory role of immunocytes in oxidative stress. Co-administration of *N*-acetylcysteine (3.5 mmol/kg, orally) with phosphamidon attenuated the adverse effects of phosphamidon. These findings suggest that oral *N*-acetylcysteine treatment exerts protective effect and attenuates free radical injury and immune dysfunction caused by subchronic phosphamidon exposure.

Key words: Cytokines; Antioxidant; Organophosphate; Oxidative stress; Immunotoxicity

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Phosphamidon (2-chloro-2-diethylcarbamoyl-1-methylvinyl dimethyl phosphate) is a broad spectrum, systemic and contact organophosphate insecticide used extensively to protect crops. The primary mechanism of action of organophosphate compounds is neurotoxicity. They are designed to be effective inhibitors of acetylcholine esterase through interaction of nucleophilic active site serine of the enzyme to form a phosphorylated enzyme derivative (1). Exposure of the general population to this pesticide may occur through consumption of foods treated with pesticides or harvested before pesticide residues have

declined to acceptable levels, from contact with treated areas during structural applications or by drift from aerial spraying. Several cases of suicidal or occupational poisoning have been reported (2). Hence, phosphamidon has generated considerable concern about its potential to cause adverse health effects in human and animals.

We have already shown that pesticide exposure results in increased lipid peroxidation coupled with altered levels of oxygen free radical scavenging enzymes and glutathione redox system in male albino rats, thereby causing oxidative stress, which may profoundly affect the

immune system (3). Recent studies on animals in our laboratory suggest that phosphamidon exposure also causes immunotoxicity (4). The mechanism leading to immunotoxicity is not known. It is possible that, like other pesticides, phosphamidon exposure also causes oxidative stress that may eventually lead to immunotoxicity.

Therefore, in the present study, we have exposed experimental animals to a low subchronic dose of phosphamidon known to cause immunosuppression in order to determine if phosphamidon can cause oxidative stress. We have used *N*-acetylcysteine (NAC), a thiolic antioxidant that acts as a precursor for the natural antioxidant glutathione and which has been shown to be a scavenger of reactive oxygen species (5), to find out if oxidative stress is indeed responsible for phosphamidon-mediated immunosuppression. If oxidative stress contributes to phosphamidon-induced immunosuppression it is logical to expect that oral administration of NAC may partially or completely attenuate immune dysfunction associated with exposure to this organophosphate.

Phosphamidon (technical grade, 94.1% purity) was obtained from Rallis (Mumbai, India). NAC, bovine serum albumin, pyrogallol, 5-5-bis-dithionitrobenzoic acid (DTNB), ovalbumin, Tween-20, and anti-rat IgG conjugated with peroxidase were obtained from Sigma-Aldrich (St. Louis, MO, USA). All other reagents used were of analytical grade and obtained either from Sisco Research Laboratory or Qualigens Fine Chemicals (Mumbai, India). Male Wistar albino rats, weighing 200-250 g, were kept under standard laboratory conditions (12-h light/dark cycle,  $20 \pm 2^\circ\text{C}$ , relative humidity,  $70 \pm 10\%$ ). Food consumption, general condition and any clinical symptom were observed daily and body weight was recorded weekly. Approval of this study was obtained from the Institutional Animal Ethics Committee. The Ethical Committee Animal Research guidelines of the University College of Medical Sciences, Delhi, India, were followed during this research.

Rats were divided randomly into four groups (8 animals per group). Group I received distilled water daily. Phosphamidon was dissolved in distilled water and administered to group II animals at a dose of 1.74 mg/kg. NAC was dissolved in distilled water and administered to group III rats at a dose of 3.5 mmol/kg. Group IV animals received both phosphamidon and NAC at the doses mentioned above. NAC was administered 60 min after phosphamidon treatment. All treatments were administered orally by gavage for 28 days.

After overnight fasting, animals were sacrificed by decapitation and heparinized blood samples were collected and processed for erythrocyte isolation. Whole blood samples were also collected and serum was separated for

various biochemical investigations. Hemoglobin concentration in hemolysate was estimated spectrophotometrically at 540 nm with Drabkin's reagent (6). Serum protein was estimated by the method of Lowry et al. (7). Tsuchihashi extract was prepared as described by Banerjee et al. (8). Malondialdehyde (MDA) level in serum (as an index of *in vivo* lipid peroxidation) was determined according to the method described by Satoh (9), using thiobarbituric acid reagent. The activity of superoxide dismutase (SOD) in Tsuchihashi extract was measured by the method of Nandi and Chatterjee (10). The unit of activity of SOD has been defined as the amount of enzyme that inhibits the auto-oxidation of pyrogallol by 50% under standard conditions. Catalase (CAT) activity in Tsuchihashi extract was determined as described by Sinha (11). Activity of SOD and CAT was expressed as U/gHb. Reduced glutathione (GSH) content in blood was measured by the method of Beutler et al. (12) using DTNB. The concentration of blood GSH was reported as mg/gHb.

For immunological tests, rats were immunized with  $2 \times 10^9$  sheep red blood cells (*ip*, in 0.5 mL saline), or with ovalbumin (*sc*, 3 mg dissolved in 0.2 mL saline) mixed with an equal volume of Freund's complete adjuvant. Sterile liquid paraffin (5 mL) was injected (*ip*) in rats immunized with ovalbumin 48 h before terminating the exposure.

Humoral immune response was assayed by estimating the serum antibody titer to ovalbumin using the hemagglutination technique (13). The antibody titer is reported as  $\log_2$  of the reciprocal of the first dilution in which no visible agglutination was observed. Leukocyte migration inhibition (LMI) and macrophage migration inhibition (MMI) assays were carried out as described by Seth et al. (13). Leukocytes from ovalbumin-immunized rats were isolated from blood following dextran precipitation and were used for the LMI test. Similarly, the peritoneal exudate cells from ovalbumin-immunized rats were used in the MMI assay. Results are reported as percent migration. Cytokines (TNF- $\alpha$  and IFN- $\gamma$ ) were measured in serum using ELISA kits obtained from Diaclone Research (Besancon Cedex, France).

All data are reported as mean  $\pm$  SD for 8 animals per group. Data were analyzed by ANOVA using the SPSS version 5 statistical program and comparisons between the various treated groups were carried out by the Tukey multiple comparison test. Comparisons with  $P < 0.05$  were considered to be statistically significant.

The dose of phosphamidon that was used is subchronic (1/10th of LD<sub>50</sub> for rats) and at this dose the treated animals did not show any overt sign of neurotoxicity, or any other observable toxic symptom.

The effects of phosphamidon, NAC and NAC with

phosphamidon on lipid peroxidation, antioxidant enzymes, and glutathione content are reported in Table 1. Our results clearly show that phosphamidon exposure induced oxidative stress. There was a significant increase in MDA and decreased GSH levels as well as a concomitant decrease in activity of antioxidant enzymes SOD and CAT. Co-treatment with NAC, a potent antioxidant, reduced the level of oxidative stress. Administration of NAC alone increased the level of GSH, but did not alter MDA, SOD or CAT activity compared to control. Therefore, NAC may directly suppress oxidative stress by enhancing the production of the endogenous antioxidant glutathione, which can scavenge hydroxyl and peroxy radicals. A recent study has also shown that NAC attenuates organophosphate-induced oxidative stress in the mouse (14).

Rats exposed to phosphamidon showed a significant decrease in primary antibody titer as well as decreased LMI and MMI compared to control indicating that phosphamidon exposure suppresses both humoral- and cell-mediated

immunity (Table 2). These results are consistent with our previous reports of the suppression of immune response parameters following exposure to phosphamidon (4). The IFN- $\gamma$  level in the serum of phosphamidon-treated animals decreased significantly compared to control. Co-treatment of NAC with phosphamidon enhanced IFN- $\gamma$  significantly. NAC possibly up-regulates cell-mediated immune response, since IFN- $\gamma$  regulates Th1 differentiation. TNF- $\alpha$  increased 3-fold with phosphamidon treatment compared to control or the NAC-treated group. Co-treatment of NAC and phosphamidon significantly reduced serum TNF- $\alpha$ . TNF- $\alpha$  is known to exert stimulatory response to several immunocytes enabling them to synthesize more reactive free radicals. Therefore, an increase in TNF- $\alpha$  in response to phosphamidon may be associated with enhancement of oxidative stress that is seen in phosphamidon-treated animals.

Co-administration of NAC with phosphamidon restored antibody response as well as LMI and MMI to control

**Table 1.** Effect of phosphamidon (PM) and *N*-acetylcysteine (NAC) on serum malondialdehyde (MDA) levels, activity of antioxidant enzymes and reduced glutathione (GSH) in erythrocytes of rats.

Parameter	Groups			
	Control	PM	NAC	PM + NAC
MDA (nmol/mL)	2.91 $\pm$ 0.24	3.83 $\pm$ 0.18*	2.56 $\pm$ 0.48 <sup>+</sup>	2.97 $\pm$ 0.12 <sup>+</sup>
GSH (mg/gHb)	2.28 $\pm$ 0.08	1.25 $\pm$ 0.21*	2.64 $\pm$ 0.15**	2.15 $\pm$ 0.58 <sup>+</sup>
SOD (U/gHb)	749.16 $\pm$ 102.61	567.8 $\pm$ 24.36*	744.62 $\pm$ 69.3 <sup>+</sup>	726.38 $\pm$ 65.54 <sup>+</sup>
CAT (U/gHb)	2.43 $\pm$ 0.08	1.86 $\pm$ 0.18*	2.14 $\pm$ 0.66 <sup>+</sup>	2.17 $\pm$ 0.18 <sup>+</sup>

Rats were treated with either PM (1.74 mg/kg), NAC (3.5 mmol/kg) or PM + NAC, orally, once daily for 28 days. Control animals received distilled water. Animals were sacrificed 24 h after the last treatment. Data are reported as means  $\pm$  SD for 8 animals/group. SOD = superoxide dismutase; CAT = catalase. P < 0.05 compared to \*control and <sup>+</sup>phosphamidon-treated group (Tukey test).

**Table 2.** Effect of phosphamidon (PM) and *N*-acetylcysteine (NAC) on humoral- and cell-mediated immune response in albino rats.

Parameter	Groups			
	Control	PM	NAC	PM + NAC
Antibody titer (-log <sub>2</sub> )	8.00 $\pm$ 0.12	4.71 $\pm$ 0.51*	8.79 $\pm$ 0.39** <sup>#</sup>	7.38 $\pm$ 0.47** <sup>#+</sup>
LMI (%)	70.80 $\pm$ 1.09	25.24 $\pm$ 1.04*	76.74 $\pm$ 2.96** <sup>#</sup>	67.24 $\pm$ 2.38** <sup>#+</sup>
MMI (%)	67.16 $\pm$ 5.30	20.38 $\pm$ 0.99*	77.96 $\pm$ 3.83** <sup>#</sup>	62.88 $\pm$ 3.00** <sup>#+</sup>
TNF- $\alpha$ (pg/mL)	23.16 $\pm$ 4.00	64.19 $\pm$ 6.00*	20.11 $\pm$ 1.40** <sup>#</sup>	35.19 $\pm$ 3.88** <sup>#+</sup>
IFN- $\gamma$ (pg/mL)	55.84 $\pm$ 3.02	40.70 $\pm$ 3.21*	60.63 $\pm$ 3.51** <sup>#</sup>	51.12 $\pm$ 1.39** <sup>#+</sup>

The experimental design is described in the legend to Table 1. For immunological tests, rats were immunized with  $2 \times 10^9$  sheep red blood cells (*ip*, in 0.5 mL saline) or with ovalbumin (*sc*, 3 mg dissolved in 0.2 mL saline) mixed with an equal volume of Freund's complete adjuvant. Data are reported as means  $\pm$  SD for 8 animals/group. LMI = leukocyte migration inhibition; MMI = macrophage migration inhibition.

P < 0.05 compared to \*control, <sup>#</sup>phosphamidon- and <sup>+</sup>NAC-treated groups (Tukey test).

levels. Therefore, NAC appears to protect immune alterations resulting from phosphamidon exposure. Administration of NAC alone also enhanced both humoral- and cell-mediated immune responses. The mechanism by which NAC prevents phosphamidon-mediated immunosuppression is not known. Since NAC is a known antioxidant (5), it is, therefore, possible that the prevention of phosphamidon-induced oxidative stress by NAC is associated with the prevention of phosphamidon-mediated immunosuppression because pesticide-mediated oxidative stress is one of major causes of pesticide-induced immunotoxicity. Increased oxygen free radical generation due to pesticide exposure can exert deleterious effects on immune function since oxygen free radicals have many molecular and cellular targets in the immune system (3).

The insecticide activity of phosphamidon is through neu-

rotoxicity (1). Previously, we have shown depletion of acetylcholine esterase (AChE), a biomarker of neurotoxicity, as a result of phosphamidon exposure and in pesticide poisoning cases (4,15). Several authors have also demonstrated depletion of brain AChE and GSH as a result of organophosphate exposure showing a link between oxidative stress and neurotoxicity (16,17). NAC treatment ameliorates AChE and GSH levels. Recently, arsenic-induced neurotoxicity was shown to be mediated through oxidative stress and NAC treatment attenuated arsenic-induced toxicity (18).

Therefore, phosphamidon-induced oxidative stress may be the underlying mechanism of neurotoxicity and immunotoxicity and NAC administration may be of therapeutic use to combat phosphamidon toxicity. Further studies using neuronal cell need to be undertaken to understand the associated mechanism at the molecular level.

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