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Effect of Dietary Treatment with $n$-Propyl Gallate or Vitamin E on the Survival of Mice Exposed to Phosgene†

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Phosgene, widely used in industrial processes, can cause life-threatening pulmonary edema and acute lung injury. One mechanism of protection against phosgene-induced lung injury may involve the use of antioxidants. The present study focused on dietary supplementation in mice using $n$-propyl gallate (nPG)—a gallate acid ester compound used in food preservation—and vitamin E. Five groups of male mice were studied: group 1, control-fed with Purina® rodent chow 5002; group 2, fed 0.75% nPG (w/w) in 5002; group 3, fed 1.5% nPG (w/w) in 5002; group 4 fed 1% (w/w) vitamin E in 5002; and group 5, fed 2% (w/w) vitamin E also in 5002. Mice were fed for 23 days. On day 23 mice were exposed to 32 mg m$^{-3}$ (8 ppm) phosgene for 20 min ($640$ mg m$^{-3}$$\cdot$min$^{-1}$) in a whole-body exposure chamber. Survival rates were determined at 12 and 24 h. In mice that died within 12 h, the lungs were removed and lung wet weights, dry weights, wet/dry weight ratios, lipid peroxidation (thiobarbituric acid reactive substances, TBARS) and glutathione (GSH) were assessed. Vitamin E had no positive effect on any outcome measured. There was no significant difference between 1.5% nPG and any parameter measured or survival rate compared with 5002 + phosgene. However, dietary treatment with 0.75% nPG significantly increased survival rate ($P < 0.002$) and lowered TBARS ($P < 0.05$) compared with 5002 + phosgene at 12 h after exposure. Mice fed 0.75% nPG had a lower wet/dry wt ratio compared with those fed 1.5% nPG and a significantly increased lung tissue GSH 36%, compared with the 5002 + phosgene group. In conclusion, dietary treatment with a low level of the antioxidant nPG protected mice by decreasing lipid peroxidation and increasing lung tissue GSH. The higher level of nPG and both levels of vitamin E diets were ineffective, suggesting that a ceiling threshold level of antioxidants in lung tissue is required for survival against phosgene-induced lung injury.

INTRODUCTION

Phosgene (COCl$_2$) is used extensively as an industrial chemical intermediate for the production of isocyanates, pesticides, plastics and polyurethanes. Large-scale usage poses a risk for environmental, accidental and/or occupational exposure. Phosgene poisoning in man is associated with the development of latent non-cardiogenic pulmonary edema, which may occur 6–24 hours after exposure. Severe inflammation and bronchospasm may also occur in moderate to high exposures. Phosgene has been directly responsible for industrial fatalities. Exposure to phosgene can cause pathophysiological changes in the lung. Experimentally, phosgene has been shown to increase substantially pulmonary vascular permeability, increase both arachidonic acid mediator production and lipid peroxidation and alter the glutathione redox state.

Treatment of phosgene-induced acute lung injury generally has involved compounds given just prior to exposure or as post-treatments. In a previous study, we have shown that dietary pretreatment with two levels of butylated hydroxynonyl (BHA) enhanced survival in an identical mouse exposure model. To investigate further the effect of dietary antioxidant supplementation, two well-known compounds were chosen: $n$-propyl gallate (nPG) and vitamin E. The antioxidant effects of nPG are not as widely known as those of BHA, butylated hydroxytoluene (BHT) or vitamin E. $n$-Propyl gallate is used extensively as an antioxidant in fats and oils to prevent rancidity and spoilage. It is also used in food preservation and in cosmetic formulations. Its permitted use level in the USA is 0.02%. Experimentally, nPG has been shown to be protective against free radical injury in vitro in lung reperfusion models and when given as a dietary supplementation in in vivo studies. Although nPG has been shown to be toxic to mitochondria, its protective effects have been ascribed to its capacity to enhance significantly the antioxidant enzymes catalase.

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and glutathione reductase.\textsuperscript{10} \textit{n}-Propyl gallate protects against CCl\textsubscript{4}-induced liver injury by inhibiting the microsomal enzyme system and lipid peroxidation processes.\textsuperscript{12} As with any antioxidant, not all effects are of a beneficial nature. Studies by Nakagawa have shown that gallates and their related esters cause hepatocyte toxicity and DNA fragmentation.\textsuperscript{13,14} Overall, however, research with gallates indicates that they may be used safely as antioxidants.\textsuperscript{15}

The advantageous uses of vitamin E as an antioxidant are too numerous to mention. Vitamin E may be the most widely studied lipid-soluble antioxidant.\textsuperscript{16–18} It is a well-known free radical chain-breaker and is thought to function as a membrane stabilizer.\textsuperscript{19} There have been many studies addressing the protective effects of vitamin E in numerous \textit{in vitro} and \textit{in vivo} models.\textsuperscript{20,21} For example, it has been demonstrated in lung studies that vitamin E can protect against hyperoxia-induced vascular paralysis in the rabbit\textsuperscript{22} and cellular O\textsubscript{2} and NO\textsubscript{2} toxicity.\textsuperscript{23} Vitamin E caused no increase in acute mortality following copper sulfate challenge when fed to rats for 60+ days.\textsuperscript{24} Despite all the positive results obtained from the use of vitamin E, there is the pro-oxidant side to consider.\textsuperscript{25} High doses of vitamin E have been shown to enhance the growth of hepatic lesions in mice.\textsuperscript{26} Furthermore, the tumor promotion qualities of topically applied vitamin E in the murine model have been demonstrated.\textsuperscript{27}

The purpose of this study is to investigate the effects of two additional well-known dietary antioxidants on survival rates and gravimetric and lung tissue antioxidant status as indicators of phosgene exposure injury.

**MATERIAL AND METHODS**

Male CD-1 mice (Charles River, Wilmington, MA) were fed a diet consisting of commercially available rodent chow: Purina\textsuperscript{8} 5002 (Richmond, IN). This diet was used as the control food source for the entire study. To this diet, nPG was added to a final concentration of 0.75\% or 1.5\%. In a separate and duplicate study, vitamin E was added to the 5002 diet at 1\% or 2\%. The concentrations of all diet levels were quantified by the manufacturer. Male CD-1 mice (Charles River, Wilmington, MA) were fed either control 5002 or antioxidant diets for 23 consecutive days prior to exposure. All mice were weighed daily (except weekends) to track their eating habits. Temporal changes in mouse body weights can be seen in Figs 1 and 2.

Five test groups were used for this study. Mice weighing 25–30 g were exposed in a dynamic whole-body exposure system to an equivalent concentration of phosgene as described above. Group 5 was fed either 1\% or 2\% vitamin E and exposed to room air for 25 min.

All elements of exposure were performed in an approved laboratory fume hood. A 10\% phosgene–90\% N\textsubscript{2} (Matheson Gas Products, Baltimore, MD) gas was metered through a Tylan\textsuperscript{8} mass flow controller (Tylan Corp., Torrance, CA) at a rate dependent on the desired concentration. This was mixed with room air and then passed through an infrared spectrometer (Miran 1A, Foxboro Co., Sharon, MA). The Miran 1A was equipped with a real-time analog output. The exposure occurred in a Plexiglas\textsuperscript{8} chamber (25 cm in height × 28 cm in diameter) with a total volume of 15.81 at a flow rate of 20.1 min\textsuperscript{-1}. Exposure to phosgene was for 20 min, followed by a 5-min room air washout. Outflowing gas from the chamber was passed through a second Miran 1A unit to determine the concentration of phosgene exiting the chamber. Effluent from the hood was passed first through an M18 filter and then through standard activated-charcoal fume-hood filters. Under these conditions, the coefficient of variation of exposure to phosgene has been calculated to be 3.7 ± 0.5\% (n = 30).

Mice were observed for 12 h from the start of exposure and again at the 24-h time point after the start of exposure. At these time points, survival rates were determined (Table 1). Mice that expired within 12 h were weighed immediately and necropsied for their lung tissue. Lung edema was estimated from the wet/dry ratio. The wet left lung was placed in a tared planchet, weighed, and placed in an oven at 100°C and reweighed 8 days later for dry weight determination. The entire right lung was frozen quickly in liquid nitrogen and stored under nitrogen at −80°C for measurements of glutathione (GSH) and thiobarbituric acid reactive substances (TBARS). All left lung gravimetric data are from mice that expired up to 12 h after the start of exposure. There are no gravimetric parameters or GSH data from lung tissue of mice that died between 12 and 24 h because these tissues were not fresh. There were no deaths associated with the combination of air exposure and any diet level of nPG or vitamin E and there were no changes between the control diet and nPG or vitamin E levels for any parameter studied following air exposure. As a result, air-related data are not included. All mice described herein were exposed to phosgene.

**Glutathione assay**

The assay for total GSH followed that of Tietze.\textsuperscript{27} Because the mass of individual mouse lungs was small, three to four mouse lungs were pooled for analysis. Two hundred milligrams of lung tissue from mice was pulverized in liquid N\textsubscript{2}. Tissue was then diluted 1:10 in 1 M perchloric acid–2 mM EDTA to precipitate proteins, homogenized for 30 s using a polytron tissue tearator (PT 10-3S Kinematic, Brinkman Ins. Westbury, NY) and centrifuged at 2000 g for 10 min at 25°C. Aliquots (200 µl) of tissue supernatant were analyzed for total GSH by catalytic conversion to GSH by glutathione reductase in the presence of Ellman’s reagent (5,5’ DTNB (dithiobis 2-nitrobenzoic acid)) in 100 mM sodium phosphate–5 mM EDTA buffer (pH 7.0).

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Figure 1. The temporal effects of a 3-week dietary regimen of nPG on mouse body weight.

Figure 2. The temporal effects of a 3-week dietary regimen of vitamin E on mouse body weight.

Table 1. The effect of phosgene exposure on 12- and 24-h mouse survival rates

<table>
<thead>
<tr>
<th>Mouse survival rates</th>
<th>12-h (alive/total)</th>
<th>24-h (alive/total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control diet (5002)</td>
<td>36% (11/30)</td>
<td>23% (7/30)</td>
</tr>
<tr>
<td>0.75% nPG (5002) + phosgene</td>
<td>85% (17/20)**</td>
<td>55% (11/20)*</td>
</tr>
<tr>
<td>1.5% nPG (5002) + phosgene</td>
<td>60% (12/20)</td>
<td>25% (5/20)</td>
</tr>
<tr>
<td>1% Vitamin E (5002) + phosgene</td>
<td>25% (5/20)</td>
<td>10% (2/20)</td>
</tr>
<tr>
<td>2% Vitamin E (5002) + phosgene</td>
<td>25% (5/20)</td>
<td>10% (2/20)</td>
</tr>
</tbody>
</table>

*Dietary pretreatment with nPG, but not vitamin E, significantly enhanced survival rates at 12 h when compared with the phosgene-exposed control diet (5002) group. Data were analyzed by the \( \chi^2 \) test: \( *P \leq 0.05 \) and \( **P \leq 0.002 \) vs 12-h phosgene-exposed control diet group.

Thiobarbituric acid reactive substances (TBARS)

Lung tissues from mice were analyzed for TBARS, an indicator of lipid peroxidation. One hundred milligrams of pooled lung tissue was homogenized in 900 \( \mu l \) of 1.15% cold KCl solution along with 15 \( \mu l \) of 0.3% butylated hydroxytoluene (BHT) in ethanol. A 400-\( \mu l \) aliquot of this 10% homogenate was mixed with 3 ml of trichloroacetic acid (15% w/v in 0.25 N HCl) and 1 ml of thiobarbituric acid solution (0.37% w/v in 0.25 N HCl) in stoppered 10-ml test tubes. These tubes were vortexed and centrifuged for 15 min at 2000 g to pellet the protein. Supernatants were removed and boiled for 1 h at 100°C and then chilled on ice. After cooling, chromagen was removed using 4 ml of 4-butanol, vortexed and centrifuged at 1000 g for 20 min. The organic phase was collected and absorbance read at 532 nm against a sample blank in a UVVIS spectrophotometer. The TBARS concentrations were calculated using an extinction coefficient of 1.56 \( \times 10^5 \) M\(^{-1}\) cm\(^{-1}\). The assay described above combined the techniques from several sources. \( \text{28-30} \) To check the accuracy of the assay, standards using 1,1,3,3-tetraethylpropane processed in a similar manner were run. A >90% recovery of a TBARS-like compound was observed using the combined procedures described above. Both GSH and TBARS were stan-
standardized to the protein concentration as determined by the Lowry method.

Statistics

Data were analyzed using a one-way ANOVA. If significant differences were found, a Duncan's multiple range post hoc test was run to assess significance levels between groups. The significant level was set at $P \leq 0.05$. Survival data were analyzed using the $\chi^2$ test. Where indicated, all data are expressed as mean $\pm$ SEM.

RESULTS

Figures 1 and 2 show the temporal effects of a 23-day diet of either nPG or vitamin E on mouse body weights. There were no differences in weight gain between control 5002 diet, 0.75% nPG or 1.5% nPG diets during the study. Mouse weights for both vitamin E diet levels were similar to controls.

Dietary treatment with nPG at either the 0.75% or 1.5% levels or vitamin E at the 1% or 2% levels did not significantly decrease lung wet weight compared with the 5002 diet + phosgene exposure (data not shown). In addition to the lack of effect on wet weight, neither nPG nor vitamin E had any significant effect (decrease) on left lung dry weight after phosgene inhalation (data not shown). As a result of these two parameters being largely unaffected by antioxidant diets, the ratio of wet lung weight/dry lung weight (W/D) was not different when compared with the 5002 diet + phosgene (Fig. 3). However, 1.5% nPG + phosgene did cause a slightly higher, but insignificant, W/D ratio compared with 0.75% nPG + phosgene.

The most significant changes in phosgene-exposed mice between antioxidant diet treatment and control 5002 diet + phosgene occurred with TBARS and GSH. Phosgene exposure caused a significant rise in the lipid peroxidation indicator TBARS. In the control 5002 diet + phosgene TBARS was increased by about 40% (33.3 $\pm$ 1.9 nmol mg$^{-1}$ protein) compared with the 0.75% nPG diet + phosgene (24.5 $\pm$ 1.9 nmol mg$^{-1}$ protein, $P = 0.05$). Mice treated with 1.5% nPG had a TBARS level very near that of the control 5002 diet + phosgene group (Fig. 4). Exposure to phosgene significantly depleted lung tissue GSH (7.3 $\pm$ 0.8 nmol mg$^{-1}$ protein) when compared with that of the 0.75% nPG diet group (12.1 $\pm$ 2 nmol mg$^{-1}$ protein, $P \leq 0.05$). Although the 1.5% nPG diet increased GSH by 40%, this was not a statistically significant amount. Neither diet level of vitamin E had any effect on lung tissue GSH compared with any other group (Fig. 5). The TBARS was not run on vitamin E fed mice because of the lack of effect of protection on survival rates, lung gravimetric data and GSH levels.

Dietary pretreatment with nPG did enhance survival rates (Table 1). This occurred at both nPG diet levels, but it was only significant at the 12-h 85% ($P < 0.002$) and 24-h 55% ($P \leq 0.05$) time points when comparing 0.75% nPG + phosgene against the control 5002 diet + phosgene. Although at 12 h 1.5% nPG + phosgene did produce three survivors for every five mice, neither the 1.5% nPG level nor the vitamin E diet had any positive impact on survival rates.
DIETARY ANTIOXIDANTS AS INDICATORS OF PHOSGENE INJURY

Previously, we have had success using a 3-week BHA antioxidant diet to ameliorate the effects of phosgene-induced lung injury. Success was defined as the capacity of the diet to enhance survival rates in mice. The primary factors in the success with BHA were that it drastically enhanced lung tissue GSH and reduced lung wet weight and lung wet weight/body weight ratio. All three of these components may have been responsible for the phenomenal survival rates observed after BHA feeding. However, in the present study it appears that, except for lung tissue GSH and TBARS, neither nPG nor vitamin E had any effect on lung tissue gravimetric parameters.

Butylated hydroxyanisole, vitamin E, and nPG have the capacity to act as free radical scavengers in biological systems. n-Propyl gallate, a synthetic polyhydric phenol, is a member of the gallic acid ester family. Propyl gallates are good scavengers of electrophilic radicals and O₂⁻. n-Propyl gallate has also been shown to inhibit lipoxygenase and cyclooxygenase products. It is amphipathic and can readily accumulate near the surface of cell membranes and is thought to prevent the oxidation of α-tocopherols. Propyl gallate compounds have been shown to scavenge oxyrdicals such as O₂⁻ and ·OH formed enzymatically from xanthine oxidase and non-enzymatically. These biochemical functions may explain, in part, the reason for its success in the present study where 0.75% nPG increased survival rates at both 12 and 24 h. This dietary level also prevented depletion of GSH and lipid peroxidation. Previously, when GSH levels were maintained and TBARS formation kept at a minimum, animals tended to manage phosgene toxicity through the reduction of pulmonary edema formation. What cannot be explained is that survival is not dependent upon the extent of lung tissue water content. Reduction of pulmonary edema formation has been shown to be a major effect of protective compounds against phosgene toxicity. n-Propyl gallate inhibits lipoxygenase products such as leukotrienes generated via arachidonic acid oxidation. It has been demonstrated in rabbits that attenuation of leukotriene production, after phosgene inhalation, drastically reduced pulmonary edema formation. Therefore, based on these earlier results, it is unlikely that leukotrienes have a major role in the present injury model. However, it must kept in mind that we cannot negate species/response effects following exposure to phosgene. Moreover, nPG’s effect on GSH levels could be the result of its capacity to enhance the levels of the antioxidant enzyme system, such as catalase and glutathione reductase. Feeding fruitflies (Zaprinous paravittiger) nPG significantly increased both enzymes, which serves to protect against free radical injury caused by hydrogen peroxide formation in addition to aiding in maintaining GSH levels. A recent publication by Ahmad et al. shows that green tea gallates modify the response of cancer cells by imparting a differential dose-based inhibition of NFκB. The translocation of active NFκB to the cell nucleus results in increased inflammatory mediator production, such as TNF-α, IL-1 and IL-6, etc. In the present study, it may be that feeding mice nPG for 3 weeks suppressed the inflammatory response but could not prevent the severe physicochemical type of injury associated with phosgene exposure.

The failure of 1.5% nPG to enhance survival rates and GSH and TBARS levels may be related to the fact that there is a ceiling threshold for effective diet supplementation with nPG. It is known that ca. 70% of the ingested dose is absorbed in the intestines. Although this study does not address this issue, it could be that there is a saturation kinetics process operational here that prevented much of the nPG from being absorbed and, as a result, it is eliminated via the fecal route. We were surprised by the results from the vitamin E diet treatment. In fact, mortality rates (Table 1) are much higher in both vitamin E + phosgene diet groups than in the phosgene alone group, suggesting an additive effect of combined toxicities of vitamin E and phosgene. Vitamin E has been studied extensively in many animal, tissue and cell models and has repeatedly demonstrated some degree of protection against free radical-mediated injury.

There may be several reasons for the inability of vitamin E to protect against phosgene-induced lung injury. First, the dietary levels used in the present study may have caused other systemic problems not quantified in the present study. Kolaja and Klaunig have shown in mice that a 450 mg kg⁻¹ diet of vitamin E enhanced the growth of hepatic lesions. The present work used dietary levels well beyond this level. However, after 3 weeks of diet there were no outward signs of toxicity based on the rate of body weight gain (Fig. 2). Second, vitamin E can act as a prooxidant if it is overwhelmed in a free radical environment and cannot be reduced to regenerate vitamin E by water-

Figure 5. Dietary pretreatment with 0.75% nPG significantly prevented phosgene-induced depletion of right lung GSH concentration when compared with phosgene-exposed 5002 diet controls and 1.5% nPG, P < 0.005 (**) and P < 0.025 (*) respectively. Sample sizes are: control diet 5002, n = 8 lungs; 0.75% nPG + phosgene, n = 3 lungs; 1.5% nPG + phosgene, n = 4; 1% vitamin E, n = 15; 2% vitamin E, n = 15 lungs. Data are expressed as the mean ± SEM.
soluble vitamin C\textsuperscript{24} or ubiquinone,\textsuperscript{24} both of which can relieve the system of the potentially reactive tocopherol semiquinones. During the scavenging process, vitamin E forms a long-lived chromanoyx radical that has a low decay rate.\textsuperscript{41} If vitamin E cannot be reduced back to its native form via vitamin C, then the chromanoyx radical may become potentially destructive in biomembranes. Cysteine may also be required for the regeneration of vitamin E.\textsuperscript{25} As is seen in Fig. 5, GSH levels are reduced by phosgene, which may affect this regeneration pathway because GSH is the storage form of cysteine. Third, Chan has shown that vitamin E enrichment can enhance the activity of phospholipase A2, which can give rise to the production of lipoxygenase and cyclooxygenase products of the arachidonic acid (AA) cascade.\textsuperscript{43,44} Fourth, we are also led to the conclusion that there must be a basic explanation as to why nPG or BHA increases the survival rates of mice exposed to phosgene but vitamin E does not. Could it be that the structure has a role in the protection observed with BHA\textsuperscript{8} and nPG? Figure 6 shows the relative chemical structures of the antioxidants used in this and an earlier investigation using BHA.\textsuperscript{8} There have been studies that have investigated these relationships.

Kagan et al. studied various forms of \(\alpha\)-tocopherol on erythrocyte, sarcoplasmic reticulum and platelet membrane peroxidation.\textsuperscript{45} The results of their study indicated that although \(\alpha\)-tocopherol derivatives without hydrocarbon tails posses a high antioxidant capacity, they could be toxic and destructive to lipid membranes by acting as mixed-function oxidase activators. Antosiewicz et al. studied the effects of various hydrocarbon chain lengths of nitroxide compounds on carbonyl and TBARS formation and compared these effects with vitamin E and its low-molecular-weight water-soluble analog Trolox.\textsuperscript{46} The results indicated that the shortest nitroxide compound and Trolox inhibited TBARS and carbonyl formation the greatest, respectively. However, these effects are reflective of several factors, i.e. protein versus lipid oxidation and/or lipid layer versus cytosolic sites of activity. Studies with vitamin E have indicated that the structure of vitamin E may be its own worst enemy. Niki et al. have shown that the phytol tail side-chain of vitamin E actually suppresses its transfer between liposomal membranes, affecting its capacity to act effectively as an antioxidant by protecting against lipid peroxidation.\textsuperscript{47}

In conclusion, within the confines of the present study and the previous one for BHA it seems that the more complex the chemical structure of the treatment compound, the least likely it is that mice will survive phosgene exposure. The efficiency of antioxidant protection may therefore depend upon the site of oxidant activity as well as on its size/diffusibility relationship to reach affected areas. This may lend some credibility to the speculation that structure–activity relationships may play a major role in the formulation and the ultimate testing of antioxidant diets against oxidant tissue injury.

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