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Anti-Inflammatory and Antioxidant Effects of Repeated Exposure to Cruciferous Allyl Nitrile in Sensitizer-Induced Ear Edema in Mice

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

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Background: Skin sensitizers induce allergic reactions through the induction of reactive oxygen species. Allyl nitrile from cruciferous vegetables has been reported to induce antioxidants and phase II detoxification enzymes in various tissues. We assessed the effects of repeated exposure to allyl nitrile on sensitizer-induced allergic reactions.

Material/Methods: Mice were dosed with allyl nitrile (0–200 $\mu\text{mol/kg}$), and then received a dermal application of 1 of 3 sensitizers on the left ear or 1 of 2 vehicles on the right ear. Quantitative assessment of edema was carried out by measuring the difference in weight between the portions taken from the right and left ears. We tested enzymes (superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and thiobarbituric acid reactive substances (TBARS) in ears.

Results: Repeated exposure to allyl nitrile reduced edemas induced by glutaraldehyde and by 2, 4-dinitrochlorobenzene (DNCB), but not by formaldehyde. The repeated exposure decreased levels of TBARS, a marker of oxidative stress, induced by glutaraldehyde and by DNCB, but not by formaldehyde. Allyl nitrile elevated SOD levels for the 3 sensitizers, and CAT levels for formaldehyde and DNCB. Allyl nitrile also increased GPx levels for formaldehyde and DNCB, but not for glutaraldehyde. The reduced edemas were associated with changes in oxidative stress levels and antioxidant enzymes.

Conclusions: Repeated exposure to allyl nitrile reduced allergic reactions induced by glutaraldehyde and by DNCB, but not by formaldehyde. This reduction was associated with changes in ROS levels and antioxidant enzyme activities.

MeSH Keywords: **Allyl Compounds • Antioxidants • Edema • Haptens • Skin Abnormalities**

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Background

The skin, a major site of defense against pathogenic agents, is exposed to a large number of environmental chemicals, some of which induce allergic reactions (sensitizers). The potential for skin sensitization from workplace chemicals is an ongoing concern. To date, great efforts have been made to identify and quantitatively assess skin sensitizers [1,2], in view of the risk of unexpected exposure to identified and unidentified sensitizers in the workplace.

Skin sensitizers induce allergic reactions through the induction of reactive oxygen species (ROS) [3], a major source of toxicity [4]. After absorption, skin sensitizers induce oxidative stress in keratinocytes, leading to ROS formation. In the mouse ear swelling test, the sensitizer 2,4-dinitrochlorobenzene (DNCB) has been shown to induce a significant increase in ear thickness in *Nrf2*^{-/-} compared with wild-type mice [5]. In a study on the mechanism involved in particulate matter contributing to the increased incidence of asthma and allergic conditions [6], H₂O₂ production was significantly higher in particulate-stimulated *Nrf2*^{-/-} dendritic cells than in the *Nrf2*^{+/+} counterparts. This indicates an important role for *Nrf2* in skin sensitization.

Nrf2 activation is known to induce antioxidants and phase II detoxification enzymes, and can be achieved by the intake of cruciferous vegetables. Recent studies have shown that cruciferous vegetables contain glucosinolates relevant to human health [7–10]. Sinigrin, one of the glucosinolates, is found in Brussels sprouts, horseradish, mustard, and broccoli [11,12]. The hydrolysis of sinigrin by the plant enzyme myrosinase results in the generation of bioactive compounds, including allyl isothiocyanate and allyl nitrile [7], and these products are thought to be responsible for a positive effect on health. Isothiocyanates have been shown to induce phase II enzymes [8,13,14], while allyl nitrile induces enzymes in the stomach, small intestine, urinary tract, kidneys, lungs, rectum, and brain [10,15,16]. Although allyl nitrile appears to upregulate the enzymes in the body, little is known about its effect on the skin.

In the present study, we hypothesized that allyl nitrile protects against sensitizer-induced allergic reactions to some extent. To test this, we examined the relationship between inflammation (mouse ear swelling) and antioxidant defense in the skin of mice that were exposed to allyl nitrile and then received a dermal sensitizer on the ear. We focused on oxidative stress and the antioxidants superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx): SOD catalyzes the dismutation of the superoxide anion to molecular oxygen and H₂O₂, playing a crucial role in the cellular antioxidant defense system; CAT efficiently promotes the conversion of H₂O₂ to water and molecular oxygen; and GPx scavenges H₂O₂ and organic hydroperoxides by using glutathione as a hydrogen donor.

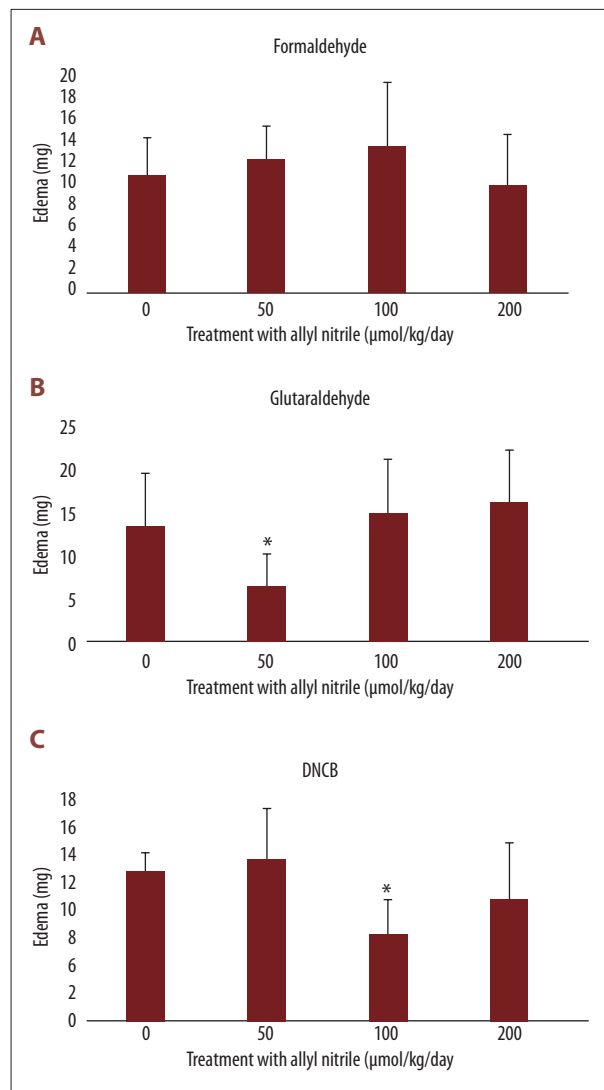


Figure 1. Edematous response to formaldehyde (A), glutaraldehyde (B), and DNCB (C) application to the ear. Values represent mean ±SD for 6 mice. Values with an asterisk are significantly different ($p < 0.05$) from the control (0 $\mu\text{mol/kg/day}$).

To explore the relationship, we used 3 skin sensitizers: formaldehyde (potency category, strong), glutaraldehyde (strong), and DNCB (extreme) [17].

Material and Methods

Materials

Allyl nitrile (3-butenenitrile, CAS 109-75-1) and DNCB (CAS 97-00-7) were purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan), and olive oil (CAS 8001-25-0) and formaldehyde solution (CAS 50-00-0) were from Wako Pure Chemical Industries

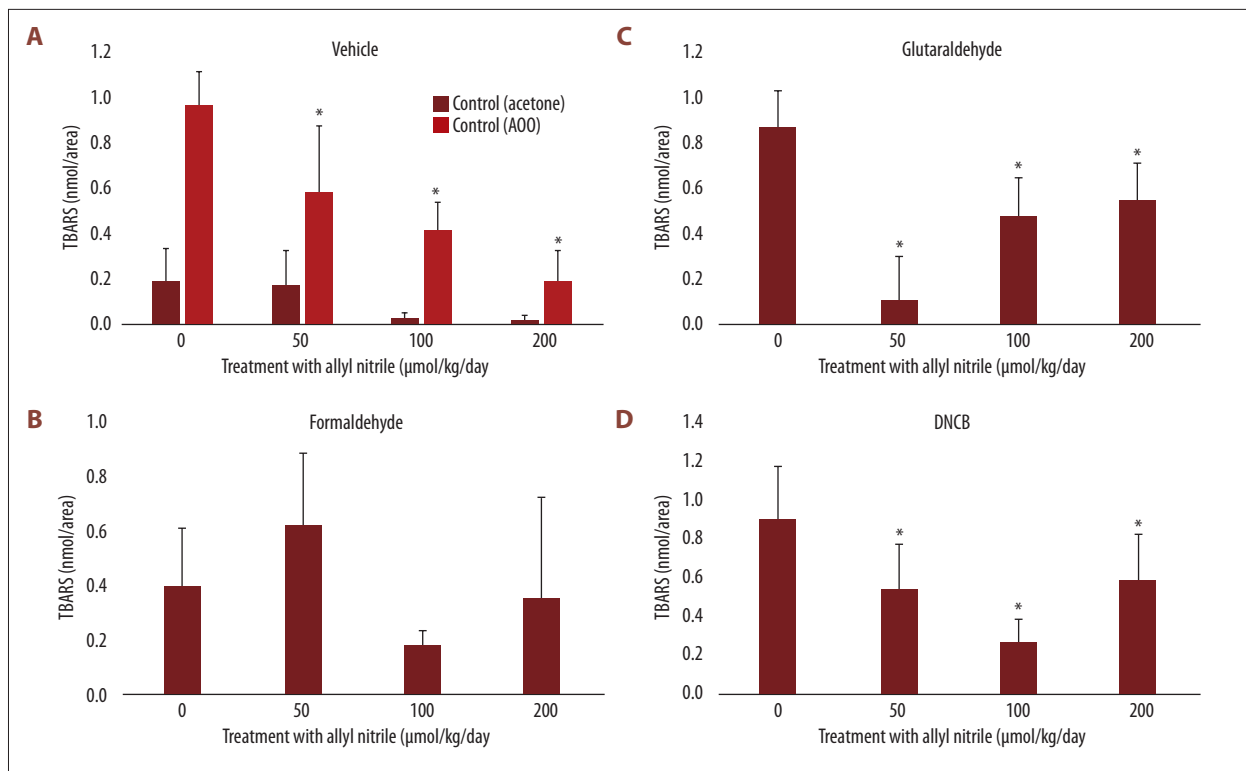


Figure 2. Thiobarbituric acid reactive substances (TBARS) levels in the ears of allyl nitrile-treated mice after application of a vehicle (acetone or AOO) (A), or the sensitizers formaldehyde (B), glutaraldehyde (C), or DNCB (D). Values (nmol/63.3 mm²) represent mean \pm SD for 6 animals. Values with an asterisk are significantly different ($p < 0.05$) from the control (0 μ mol/kg/day).

(Osaka, Japan). Acetone (CAS 67-64-1), H₂O₂ (CAS7722-84-1), glutaraldehyde (CAS 111-30-8), and other chemicals were purchased from Nacalai Tesque (Kyoto, Japan).

Animals and treatment

Animal experiments were carried out according to the Guidelines of the Committee on Animal Experimentation of Kanazawa University. Male ddY mice weighing 26–30 g were obtained from Japan SLC Co. (Shizuoka, Japan), and were maintained at 22 \pm 2°C under a 12/12 h light/dark cycle with free access to tap water and laboratory food (CRF-1; Charles River Japan Inc., Yokohama, Japan). The ddY mice, a closed colony, have been used in our previous studies [10,15,16].

Groups of 6 animals were given subtoxic doses of allyl nitrile (50, 100, or 200 μ mol/kg) or vehicle-distilled water (4 mL/kg) daily for 8 days by gastric intubation, on the basis of our previous findings [16]. On days 6, 7, and 8, the animals received 1 of 3 sensitizers (formaldehyde, glutaraldehyde, or DNCB) on the left ear and a vehicle (acetone or acetone-olive oil (4:1) [AOO]) on the right ear. The applications to the ear were 40 μ L of 20% formaldehyde in acetone, 5% glutaraldehyde in acetone, and 1.5% DNCB in AOO, acetone, and AOO. The concentration of the sensitizers was determined such that each

sensitizer would induce a similar ear edema (noted by a similar increase in weight). The animals were sacrificed on day 9 for all analyses.

Assessment of ear edema

Mice were anesthetized with 100 mg/kg sodium pentobarbital and a 63.3 mm² tissue punch was taken from both the right and left ears to evaluate edema. Quantitative assessment of edema was carried out by measuring the difference in weight between the portions taken from the right and left ears. The ears were stored at –80°C until biochemical analyses.

Tissue preparation and biochemical assays

Tissue punches were homogenized in 1 mL of buffer (0.01 M Tris-HCl buffer, pH 7.4). The homogenates were centrifuged at 10 000 g at 4°C for 20 min, and the resulting cytosolic fractions were stored at –80°C until biochemical analyses. Protein concentrations were measured according to the Bradford method [18] using bovine serum albumin as the standard.

CAT activity was measured using the method of Abei [19], using H₂O₂ as the substrate. GPx activity was measured using the spectrophotometric method of Paglia and Valentine [20], using

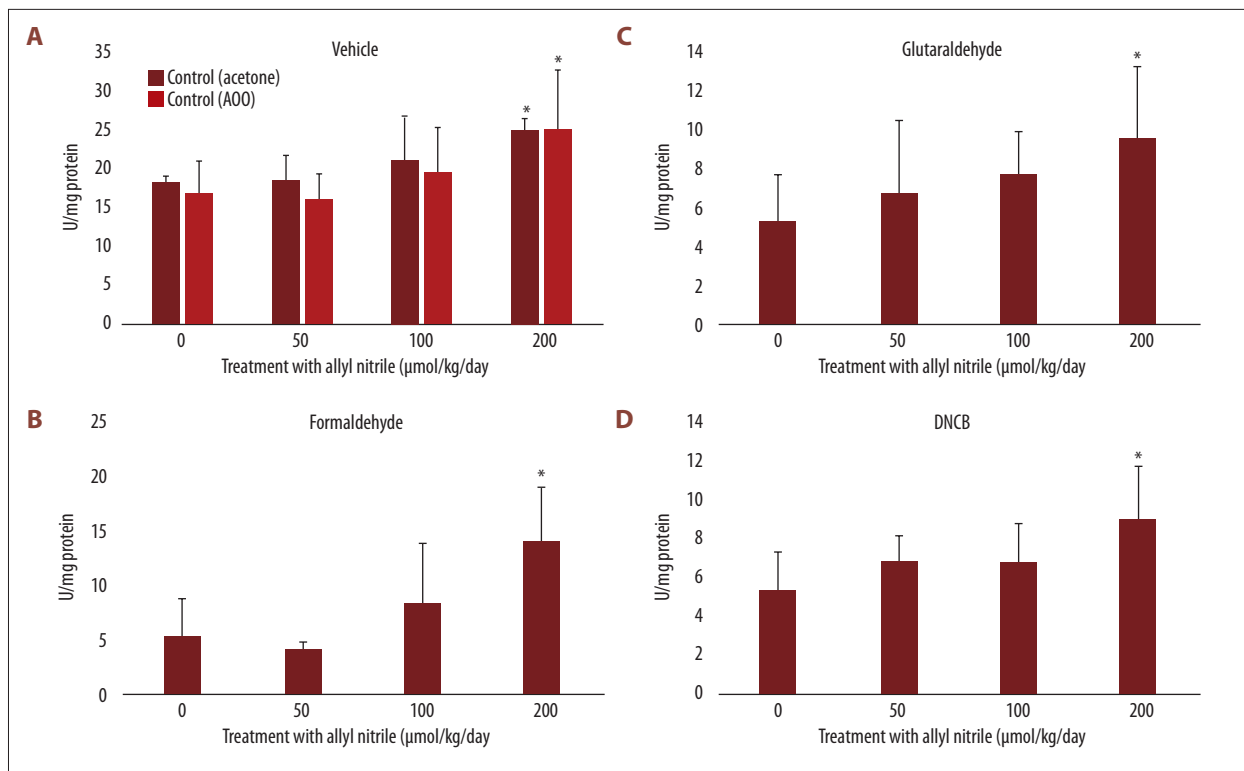


Figure 3. Superoxide dismutase (SOD) levels in the ears of allyl nitrile-treated mice after application of a vehicle (acetone or AOO) (A), or the sensitizer formaldehyde (B), glutaraldehyde (C), or DNCB (D). One unit (U) of SOD is defined as the quantity of enzyme needed to manifest a 50% dismutation of the superoxide radicals. Values represent mean \pm SD for 6 mice. Values with an asterisk are significantly different ($p < 0.05$) from the control (0 $\mu\text{mol/kg/day}$).

cumene hydroperoxide as the substrate and NADPH as the source of reducing equivalents. Thiobarbituric acid reactive substances (TBARS) and SOD levels were determined with commercial assay kits (Cayman Chemical Company, Ann Arbor, MI, USA).

Data analysis

We performed analysis of variance (ANOVA), followed by Fisher's least significant difference test for multiple comparisons. The level of significance was set at $p < 0.05$.

Results

Edematous responses to sensitizers in mice that received repeated exposure to allyl nitrile

Application of sensitizers (formaldehyde, glutaraldehyde, and DNCB) to the ear for 3 days induced edema, quantified by an increase in weight. No difference in the edema was observed between the 3 sensitizer groups and the controls (Figure 1A–1C). The repeated exposure to allyl nitrile had no observable effect on the edema induced by formaldehyde (Figure 1A). The repeated exposure, however, was correlated with a decrease

in the edemas induced by glutaraldehyde and by DNCB at the levels of 50 and 100 $\mu\text{mol/kg/day}$ (Figure 1B, 1C).

TBARS levels after sensitizer application

TBARS measurement was performed to assess oxidative stress after application of the vehicles and sensitizers. Lower levels were seen at 100 and 200 $\mu\text{mol/kg/day}$ for the vehicle acetone, and at 50, 100, and 200 $\mu\text{mol/kg/day}$ for the AOO vehicle, compared with the controls ($p < 0.05$, vs. the vehicle acetone) (Figure 2A). The TBARS levels for formaldehyde did not vary at 0–200 $\mu\text{mol/kg/day}$ (Figure 2B). The levels were decreased at 50, 100, and 200 $\mu\text{mol/kg/day}$ for both glutaraldehyde and DNCB, the lowest being at 50 and 100 $\mu\text{mol/kg/day}$ (Figure 2C, 2D). The level at 0 $\mu\text{mol/kg/day}$ for glutaraldehyde was higher than that for acetone ($p < 0.05$) (Figure 2A, 2C).

SOD levels after sensitizer application

Enhanced SOD activities were observed at 200 $\mu\text{mol/kg/day}$ for both acetone and AOO (Figure 3A). Elevated activities were recorded at 200 $\mu\text{mol/kg/day}$ for formaldehyde, glutaraldehyde, and DNCB, with lower activities at 0 $\mu\text{mol/kg/day}$ ($p < 0.05$, vs. corresponding vehicle at 0 $\mu\text{mol/kg/day}$) (Figure 3A–3D).

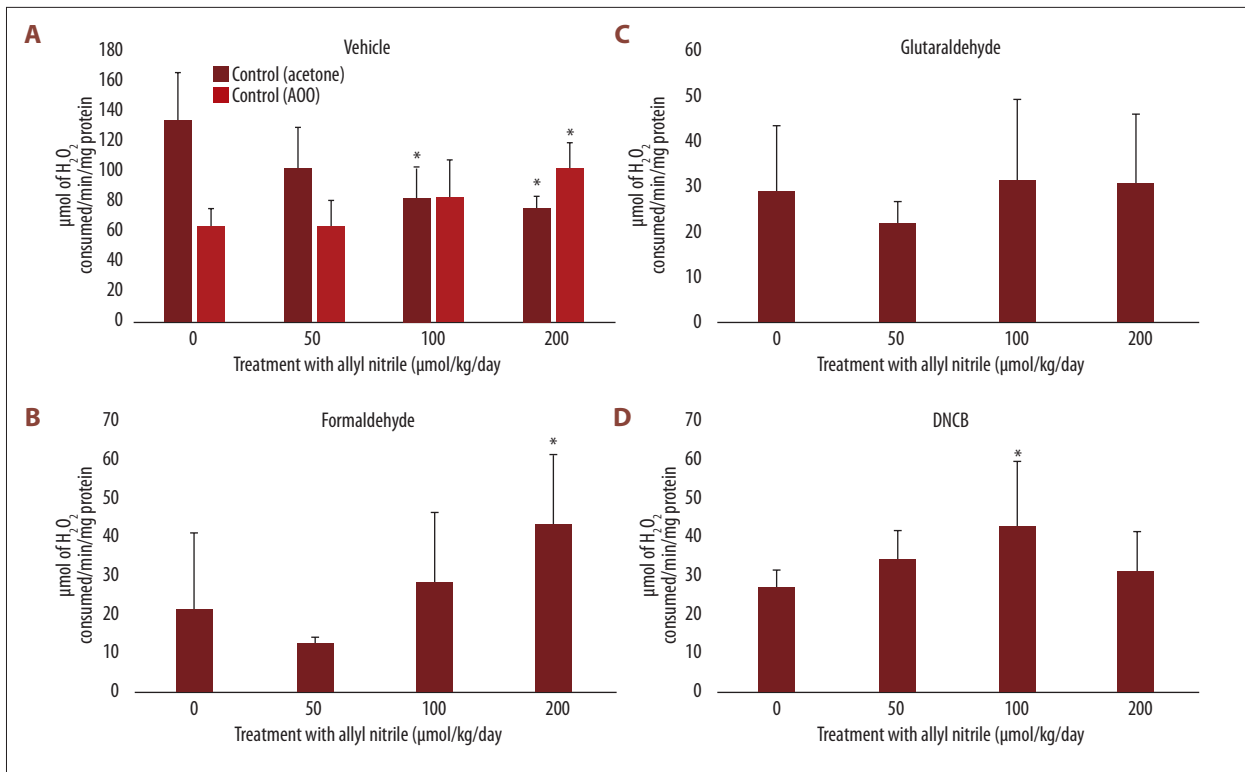


Figure 4. Catalase (CAT) levels in the ears of allyl nitrile-treated mice after application of a vehicle (acetone or A00) (A), or the sensitizer formaldehyde (B), glutaraldehyde (C), or DNCB (D). Values represent mean \pm SD for 6 mice. Values with an asterisk are significantly different ($p < 0.05$) from the control (0 $\mu\text{mol/kg/day}$).

CAT levels after sensitizer application

Decreased CAT activities were observed at 100 and 200 $\mu\text{mol/kg/day}$ for the vehicle acetone, with higher activity at 0 $\mu\text{mol/kg/day}$ ($p < 0.05$, vs. the vehicle A00), while enhanced activity was recorded at 200 $\mu\text{mol/kg/day}$ for the vehicle A00 (Figure 4A). For formaldehyde and DNCB, enhanced activities were observed at 200 and 100 $\mu\text{mol/kg/day}$, with lower activities at 0 $\mu\text{mol/kg/day}$ ($p < 0.05$, vs. corresponding vehicle at 0 $\mu\text{mol/kg/day}$) (Figure 4A, 4B, 4D). No change in activity was recorded for glutaraldehyde, with decreased levels at 0 $\mu\text{mol/kg/day}$ ($p < 0.05$, vs. the vehicle acetone at 0 $\mu\text{mol/kg/day}$) (Figure 4A, 4C).

GPx levels after sensitizer application

No change in GPx activity was recorded at 0–200 $\mu\text{mol/kg/day}$ for acetone, while an increase in activity was seen at 200 $\mu\text{mol/kg/day}$ for A00 (Figure 5A). Elevated GPx activity was observed at 200 $\mu\text{mol/kg/day}$ for formaldehyde (Figure 5B). Decreased activity was seen at 50 $\mu\text{mol/kg/day}$ for glutaraldehyde (Figure 5C), and elevated levels at 100 $\mu\text{mol/kg/day}$ for DNCB (Figure 5D).

Discussion

We showed that repeated exposure to allyl nitrile can reduce allergic reactions to glutaraldehyde and DNCB, as measured by an edematous response, depending on exposure levels. Allyl nitrile did not reduce allergic reactions to formaldehyde. The protective effect was associated with decreased oxidative stress and with modified activities of antioxidant enzymes. These results suggest that edema induced by sensitizers can be reduced by allyl nitrile-modulated antioxidants. Additionally, we noted that A00 and acetone are not interchangeable.

Edemas induced by glutaraldehyde and DNCB were decreased by exposure to allyl nitrile, but not edemas induced by formaldehyde, although almost the same degree of edema was induced by each sensitizer. Allyl nitrile may be acting by modulating the activity of antioxidant enzymes. In glutaraldehyde-treated ears, allyl nitrile at 50 $\mu\text{mol/kg/day}$ reduced edema, oxidative stress, and GPx levels, while levels of SOD increased and levels of CAT decreased. This may indicate the achievement of a particular H_2O_2 level. In DNCB-treated ears, allyl nitrile at 100 $\mu\text{mol/kg/day}$ reduced edema and oxidative stress, and enhanced CAT, GPx, and SOD levels, indicating again that a particular H_2O_2 level was achieved. In formaldehyde-treated ears, we did not find any changes in edema, oxidative stress,

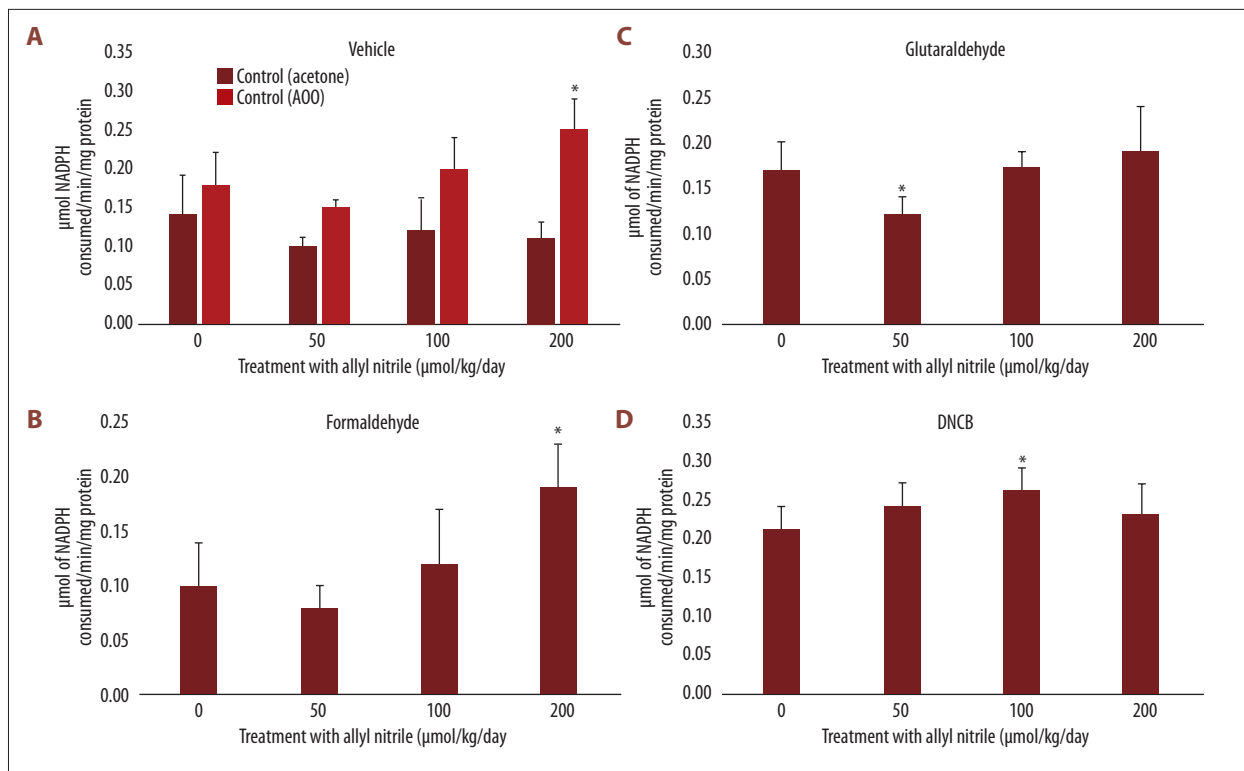


Figure 5. Glutathione peroxidase (GPx) levels in the ears of allyl nitrile-treated mice after application of a vehicle (acetone or AOO) (A), or the sensitizer formaldehyde (B), glutaraldehyde (C), or DNCB (D). Values represent mean \pm SD for 6 mice. Values with an asterisk are significantly different ($p < 0.05$) from the control (0 $\mu\text{mol/kg/day}$).

or antioxidant enzymes, except at 200 $\mu\text{mol/kg/day}$; therefore, we assume that allyl nitrile could not affect the H_2O_2 levels.

ROS in high concentrations, including H_2O_2 , promote cellular damage and tissue destruction, but recent findings suggest that ROS can act to fine-tune the inflammatory response [21]. H_2O_2 has been shown to have an effect on Nrf2, upregulating phase II enzyme expression [22]. After skin absorption, sensitizers may produce ROS. Repeated exposure to allyl nitrile may modify antioxidant activity, leading to a particular, low H_2O_2 level and reducing the edematous response.

Repeated exposure to allyl nitrile exhibited effects on CAT and GPx activities correlated by vehicle: Acetone decreased CAT activities while AOO increased them; GPx activities remained unchanged with acetone, but were enhanced under AOO. We encountered another difference between the vehicles in terms of their effects on TBARS and CAT levels in the control groups (Figure 2A, 4A): Acetone was correlated with lower levels of TBARS and higher levels of CAT, compared with AOO. The differences may be due to compounds in the olive oil [23]. Acetone and AOO have both been used as vehicles for sensitizers [17]. The vehicle itself may induce alterations in the allergic reaction process. We observed that the 2 vehicles contributed to different levels of CAT activity, which in turn would affect H_2O_2 levels.

Cruciferous vegetables have been shown in epidemiological studies to have a beneficial effect on health; their consumption may be inversely associated with the risk of various cancers [24–26]. Cruciferous allyl nitrile is thought to contribute to this preventive effect by upregulating antioxidants and phase II detoxification enzymes in various tissues [10,15,16]. The present study provides evidence that repeated exposure may be protective against allergic reactions. In addition, it has been shown that allyl isothiocyanate has an anti-inflammatory potential in cultured macrophages, which may be mediated by Nrf2 and nuclear factor κB signaling pathways, but has little anti-inflammatory activity in mice [27]. Overall, higher intake of vegetables rich in sinigrin may be beneficial to individuals exposed to common industrial chemicals in the workplace [11,12].

There are some limitations to the study. The exposure was repeated for 8 days, but the study could be repeated with a one-time exposure to allyl nitrile. This could yield valuable information on the dose and frequency required for allyl nitrile consumption to have protective benefits. Further, this study measured the immune response solely by weight of edematous tissues. A future study could measure levels of specific immune factors to determine whether these factors exert a greater effect on edema reduction than the antioxidants do.

Conclusions

Repeated exposure to allyl nitrile reduced allergic reactions induced by glutaraldehyde and by DNCB, but not by formaldehyde. This reduction was associated with changes in ROS levels and antioxidant enzyme activities. The results suggest that SOD may be involved in allyl nitrile-modified reduction of the edematous response, while CAT and GPx may be involved in

modulating the response. The 2 vehicles we used, acetone and AOO, did not result in comparable levels of oxidative stress and antioxidant enzymes.

Conflict of interest

The authors declare that they have no conflict of interest.

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