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Protective effect of N-acetyl-L-cysteine against acrylamide-induced oxidative stress in rats

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Abstract: Acrylamide (AA), used in many fields, from industrial manufacturing to laboratory work, is also formed during the heating process through the interactions of amino acids. Therefore, AA poses a significant risk for both human and animal health. This study aimed to elucidate whether N-acetyl-L-cysteine (NAC) treatment could modulate AA-induced oxidative changes in the brain, lung, liver, kidney, and testes tissues of the rat. Rats were divided into 4 groups, as the control (C), NAC [150 mg/kg intraperitoneally (i.p.)], AA (40 mg/kg i.p.), and NAC + AA groups. After 10 days, the rats were decapitated and the tissues were excised. Malondialdehyde (MDA) and glutathione (GSH) levels, and myeloperoxidase activity (MPO) were determined in the tissues, while oxidant-induced tissue fibrosis was determined using the collagen contents. Serum enzyme activities, cytokine levels, and leukocyte apoptosis were assayed in the plasma. In the AA group, GSH levels decreased significantly, while the MDA levels, MPO activity, and collagen content increased in the tissues suggesting oxidative organ damage. In the NAC + AA group, oxidant responses reversed significantly. Serum enzyme activities, cytokine levels, and leukocyte apoptosis, which increased following AA administration, decreased with NAC treatment. Therefore, supplementing with NAC can be useful when there is a risk of AA toxicity, as NAC inhibits neutrophil infiltration, balances the oxidant-antioxidant status, and regulates the generation of inflammatory mediators to protect tissues.

Key words: Acrylamide, N-acetyl-L-cysteine, oxidative changes, collagen, myeloperoxidase, leukocyte apoptosis

Introduction

Acrylamide (AA), an α, β unsaturated carbonyl compound with significantly high chemical activity, is extensively used in many fields, from industrial manufacturing to laboratory work, (1). AA gained great public and scientific interest when the World Health Organization, in 2002, published the concentrations of AA of many foods (2). Specifically, in foods containing carbohydrates and proteins, AA is formed through the Maillard reaction during the heating process by interactions of amino acids, especially asparagine, with reducing sugars like glucose (3). Moreover, most dry pet foods contain cereal grains or potatoes, and they are processed at

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high temperatures providing the conditions for the Maillard reaction. In fact, the Maillard reaction is considered desirable in the production of pet food because it imparts a palatable taste, even though it reduces the bioavailability of some amino acids, including taurine and lysine (4).

Oxidative stress and the role of reactive oxygen species (ROS) in disease and toxicity have been 2 major issues in biomedical sciences (5,6). Free radicals are continuously produced in vivo and there are number of protective antioxidant enzymes (superoxide dismutase, catalase, glutathione S-transferase, glutathione peroxidase, and antioxidant glutathione) for dealing with these toxic substances. The delicate balance between the production and catabolism of oxidants is critical for maintenance of the biological function (7).

N-acetyl-L-cysteine (NAC) is a potent antioxidant that has been used clinically for decades for the treatment of many diseases. It derives from the amino acid L-cysteine and it has been used as a chelator of heavy metal to protect against oxidative stress and prevent damage to cells (8). It plays an important role in the production of glutathione, which provides an intracellular defense against oxidative stress, and it participates in the detoxification of many molecules (9). During the last decade, numerous in vitro and in vivo studies have suggested that NAC has beneficial medicinal properties, including the inhibition of carcinogenesis, tumorigenesis, and mutagenesis, as well as the inhibition of tumor growth and metastasis (10,11). Although NAC is an excellent scavenger of free radicals and chelator of heavy metal, it remains unclear whether this compound is effective in AA-induced oxidative stress. Hence, the present study was designed to elucidate whether exposure to NAC could modulate acrylamide-induced oxidative changes in the brain, lung, liver, kidney, and testes tissues of the rat.

Materials and methods

Animals

All of the experimental protocols were approved by the Marmara University School of Medicine, Animal Care and Use Committee. Wistar albino rats of either sex (200-250 g) were housed in a room with a mean constant temperature of 22 ± 2 °C, a light/dark photoperiod of 12:12, and ad libitum standard pellet chow and water.

Experimental groups

The rats were administered Acrylamide (Merck, 800830) (40 mg/kg/day intraperitoneally (i.p.), dissolved in 0.9% NaCl) followed by either saline (AA group) or NAC (Sigma, A-7250) (at a dose of 150 mg/kg i.p., dissolved in 0.9% NaCl) (AA + NAC group) for 10 days. In the control group, saline (0.9% NaCl) or NAC (150 mg/kg i.p., dissolved in 0.9% NaCl) was injected for 10 days. Each group consisted of 6 animals.

Assays

The animals were decapitated on the 10th day, trunk blood samples were collected, and brain, lung, liver, kidney, and testis tissues were excised. Blood samples were used to analyze aspartate aminotransferase (AST), alanine aminotransferase (ALT), blood urea nitrogen (BUN), 8-hydroxy-2'-deoxyguanosine (8-OHdG), creatinine, lactate dehydrogenase (LDH) activity, tumor necrosis factor α (TNF-α), and interleukin (IL)-1β, IL-6, and IL-10. In the brain, lung, liver, kidney, and testes tissue samples, stored at −70 °C, malondialdehyde (MDA) as an end product of lipid peroxidation, glutathione (GSH) as a key antioxidant, and tissue-associated myeloperoxidase (MPO) activity as indirect evidence of neutrophil infiltration were measured. Additional tissue samples were placed in formaldehyde (10%) for the determination of collagen content. Serum enzyme activities, cytokine levels, and leukocyte apoptosis were assayed in the plasma samples.

Blood urea nitrogen and serum AST, ALT, and creatinine concentrations and LDH levels were determined spectrophotometrically using an automated analyzer (Opera Technican Bayer Autoanalyzer). Serum levels of TNF-α, IL-1β, IL-6, and IL-10 were quantified using enzyme-linked immunosorbent assay (ELISA) kits specific to the previously mentioned rat cytokines, according to the manufacturer's instructions and guidelines (Biosource Europe S.A., Nivelles, Belgium). The total antioxidant capacity in the plasma was measured using a colorimetric test system (ImAnOx, catalog no. KC5200, Immunodiagnostic AG, D-64625

Bensheim), according to the manufacturer's instructions. The 8-OHdG content in the extracted DNA solution was determined using the ELISA method (Highly Sensitive 8-OHdG ELISA kit, Japan Institute for the Control of Aging, Shizuoka, Japan). These particular assay kits were selected because of their high degree of sensitivity, specificity, inter- and intraassay precision, and small amount of plasma sample required for conducting the assay.

MDA and GSH assays

Tissue samples were homogenized with ice-cold 150 mM KCl for the determination of MDA and GSH levels. The MDA levels were assayed for products of lipid peroxidation by monitoring thiobarbituric acid reactive substance formation, as described previously (12). Lipid peroxidation was expressed in terms of MDA equivalents using an extinction coefficient of $1.56 \times 10^5\ M^{-1}\ cm^{-1}$ and the results were expressed as nmol MDA/g tissue. GSH measurements were performed using a modification of the Ellman procedure (13). GSH levels were calculated using an extinction coefficient of $1.36 \times 10^4\ M^{-1}\ cm^{-1}$. The results were expressed in μmol GSH/g tissue.

MPO activity and tissue collagen measurement

MPO, an enzyme released by activated polymorphonuclear neutrophils, is used as an indication of tissue neutrophil accumulation. Tissue MPO activity was measured using a procedure similar to that documented previously (14). Tissue samples were homogenized in 50 mM potassium phosphate buffer (PB, pH 6.0), and centrifuged at 41,400 × *g* (10 min); pellets were suspended in 50 mM PB containing 0.5% hexadecyltrimethylammonium bromide (HETAB). After 3 freeze and thaw cycles with sonication between the cycles, the samples were centrifuged at 41,400 × *g* for 10 min. Aliquots (0.3 mL) were added to 2.3 mL of reaction mixture containing 50 mM PB, o-dianisidine, and 20 mM H_2O_2 solution. One unit of enzyme activity was defined as the amount of the MPO present that caused a change in absorbance measured at 460 nm for 3 min. MPO activity was expressed as U/g tissue.

Tissue collagen content measurement

Tissue collagen was measured as a free radical-induced fibrosis marker. Tissue samples were cut with a razor blade, immediately fixed in 10% formalin in 0.1 M phosphate buffer (pH; 7.2) in paraffin, and sections measuring approximately 15 μm thick were obtained. Evaluation of the collagen content was based on the method published by Lopez de Leon and Rojkind (15), which is based on selective binding of the dyes Sirius Red and Fast Green FCF to collagen and noncollagenous components, respectively.

Evaluation apoptosis and cell death

Apoptosis and cell death was evaluated according to the method of Takei et al. (16). Erythrocytes from heparinized blood samples of the groups were discarded using flow cytometry. White blood cells were washed and resuspended in phosphate buffered saline-gel. For each apoptosis experiment, 2 tubes were prepared and 1×10^5 cells/mL were distributed into the tubes. One of the tubes was induced for apoptosis using 100 ng/mL of phorbol myristate acetate at 37 °C for 2 h, while other was incubated at the same temperature without stimulation, as a control. To demonstrate early apoptosis, the cells were washed with phosphate buffered saline following stimulation and were labeled with annexin V, according to the manufacturer's instructions (Biovision, Mountain View, CA, USA).

Statistical analysis

Statistical analysis was carried out using GraphPad Prism 3.0 (GraphPad Software, San Diego, CA, USA). Each group consisted of 8 animals. All of the data were expressed as means ± s.d. Groups of data were compared with ANOVA followed by Tukey's multiple comparison tests. $P < 0.05$ was considered as significant.

Results

As an indication of hepatic injury, the ALT and AST levels were significantly higher in the saline-treated AA group when compared with those of the control group ($P < 0.001$). NAC treatment decreased both the ALT and AST levels significantly ($P < 0.01$ and $P < 0.001$, respectively) compared with the saline-treated AA group. Serum lactate dehydrogenase (LDH) activity, as an indicator of generalized tissue damage, showed a significant increase in the AA group ($P < 0.001$) and this effect was reversed significantly by NAC treatment ($P < 0.001$, Table 1).

Table 1. Serum ALT, AST, and LDH activities and BUN, Creatinine, and 8-OHdG levels of the 4 groups.

	Control	NAC	AA	AA + NAC
ALT (U/L)	50.50 ± 5.01	51.67 ± 5.19	114.0 ± 8.90***	74.0 ± 5.46^{++}
AST (U/L)	167.3 ± 11.27	171.0 ± 9.85	390.7 ± 33.98***	214.0 ± 9.83^{+++}
BUN (U/L)	21.0 ± 2.05	22.0 ± 1.75	80.67 ± 11.42***	33.0 ± 3.72^{+++}
Creatinine (U/L)	0.45 ± 0.08	0.58 ± 0.08	2.08 ± 0.18***	0.80 ± 0.10^{+++}
LDH (U/L)	2102 ± 194.1	2120 ± 172.0	4293 ± 219.3***	2768 ± 167.0^{+++}
8-OHdG (ng/mL)	0.62 ± 0.13	0.66 ± 0.14	7.62 ± 0.84***	3.78 ± 0.28***, $^{+++}$

Data are mean ± s.d. ***P < 0.001 compared with the control group. $^{++}$P < 0.01 and $^{+++}$P < 0.001 compared with the AA group.

BUN and creatinine levels were significantly increased in the saline-treated AA group compared with those of the control group. On the other hand, NAC treatment significantly decreased these parameters in the AA group when compared with the saline-treated AA group (P < 0.001) (Table 1).

In the saline-treated AA group, the plasma TNF-α, IL-1β, IL-6, and IL-10 levels were significantly increased compared with the control group (P < 0.001). NAC treatment decreased these parameters in the AA group when compared with the saline-treated AA group (Figure 1). As an indicator of oxidative DNA damage, 8-OH dG levels were also significantly increased in the AA group and this effect was reversed significantly by NAC treatment (P < 0.001, Table 1).

In the AA group, GSH levels were decreased significantly while the MDA levels, MPO activity, and collagen content increased in the tissues, suggesting oxidative organ damage. In the NAC + AA group, all of these oxidant responses were reversed significantly (Table 2). Moreover, leukocyte apoptosis, which had increased significantly following AA administration, decreased with NAC treatment (Figures 2 and 3).

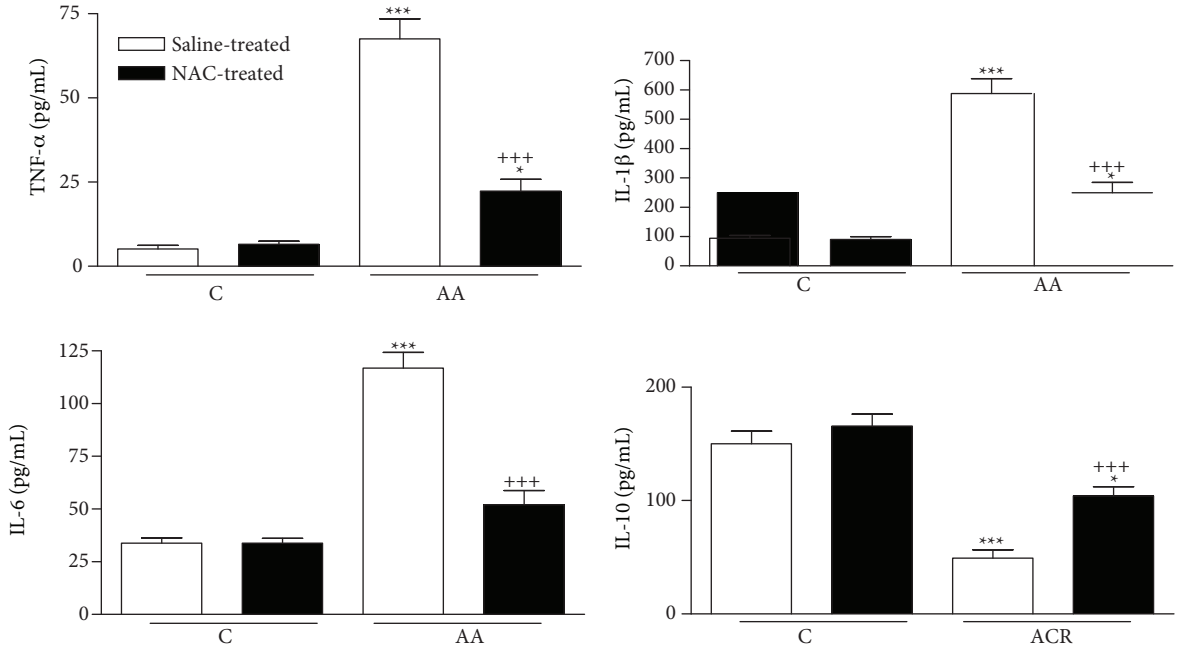

Figure 1. Serum TNF-α, IL-β, IL-6, and IL-10 levels of the groups. *P < 0.05 and ***P < 0.001: compared to the saline-treated control group. +++P < 0.001, compared to the saline-treated AA group.

Table 2. Tissue MDA, GSH, and MPO levels and collagen contents of the 4 groups.

	Control	NAC	AA	AA + NAC
MDA (nmol/g)				
Brain	27.00 ± 2.39	28.58 ± 2.12	51.28 ± 2.99***	34.77 ± 2.39^{+++}
Lung	54.78 ± 3.39	54.18 ± 3.39	87.10 ± 4.18***	68.40 ± 2.32$^{*,++}$
Liver	46.27 ± 3.78	45.63 ± 3.73	79.12 ± 4.31***	53.73 ± 4.03^{++}
Kidney	27.48 ± 2.24	26.83 ± 2.23	54.30 ± 2.62***	33.15 ± 2.38^{+++}
Testes	31.38 ± 3.13	31.03 ± 3.16	57.07 ± 2.87***	38.22 ± 2.79^{++}
GSH (μmol/g)				
Brain	0.83 ± 0.08	0.88 ± 0.06	0.46 ± 0.04***	0.89 ± 0.06^{+++}
Lung	1.93 ± 0.19	1.88 ± 0.13	0.95 ± 0.08***	1.69 ± 0.14^{++}
Liver	2.02 ± 0.08	2.10 ± 0.05	0.93 ± 0.07***	1.51 ± 0.09$^{***,+++}$
Kidney	1.91 ± 0.06	1.93 ± 0.07	0.97 ± 0.08***	1.57 ± 0.08$^{*,+++}$
Testes	1.20 ± 0.06	1.20 ± 0.07	0.52 ± 0.09***	0.93 ± 0.06^{++}
MPO (U/g)				
Brain	11.75 ± 1.36	13.05 ± 1.26	28.23 ± 2.19***	18.52 ± 1.73$^{*,++}$
Lung	24.63 ± 1.71	24.98 ± 2.43	70.50 ± 4.94***	33.22 ± 3.83^{+++}
Liver	10.67 ± 0.69	10.65 ± 0.69	30.95 ± 2.40***	16.35 ± 0.92$^{*,+++}$
Kidney	26.57 ± 2.07	26.75 ± 2.19	57.43 ± 3.29***	32.62 ± 2.10^{+++}
Testes	8.37 ± 0.82	8.92 ± 0.90	26.28 ± 2.05***	15.37 ± 1.82$^{*,+++}$
Collagen (μg/mg)				
Brain	9.60 ± 0.65	9.75 ± 0.79	20.55 ± 1.30***	13.62 ± 0.98$^{*,+++}$
Lung	16.60 ± 0.92	17.17 ± 1.01	34.50 ± 1.27***	21.22 ± 1.22$^{*,+++}$
Liver	14.75 ± 0.91	13.98 ± 1.29	30.95 ± 1.15***	20.70 ± 1.28$^{**,+++}$
Kidney	13.95 ± 0.94	14.58 ± 0.96	30.97 ± 2.27***	18.48 ± 1.16^{+++}
Testes	9.98 ± 1.23	9.83 ± 0.95	20.10 ± 1.35***	11.97 ± 0.99^{+++}

Data are mean ± s.d. *P < 0.05, **P < 0.01, and ***P < 0.001 compared with the control group. $^{+}$P < 0.05, $^{++}$P < 0.01, and $^{+++}$P < 0.001 compared with the AA group.

Discussion

In the present study, increases in MDA levels, MPO activity, and collagen content, as well as the decrease in GSH levels induced by AA were reversed by NAC treatment. These findings demonstrate that AA-induced oxidative organ damages in the brain, lung, liver, kidney, and testes tissues were ameliorated by NAC treatment. Moreover, impairments in hepatic and renal functions due to AA were also improved by NAC treatment, and plasma levels of the proinflammatory cytokines and oxidative DNA damage were reduced. These findings suggest that NAC may be protective against AA-induced oxidative injury by inhibiting neutrophil infiltration, and subsequent activation of inflammatory mediators that induce lipid peroxidation.

The spontaneous formation of AA during the cooking of food has led to its description as a cooking carcinogen (17). In addition to the evidence of AA mutagenicity (18), genotoxicity (19) and carcinogenicity (20) have also been reported. AA therefore poses a potentially significant risk both for human and animal health. However, the mechanism by which AA exposure causes cellular dysfunction in experimental animals and humans is not clear. In the present study, we provide experimental evidence in support of the role of oxidative stress in AA toxicity.

Enhancement of lipid peroxidation is a consequence of depletion of GSH to certain critical levels. AA is oxidized to glycidamide, a reactive epoxide, and undergoes conjugation with glutathione. DNA adducts from glycidamide have been reported

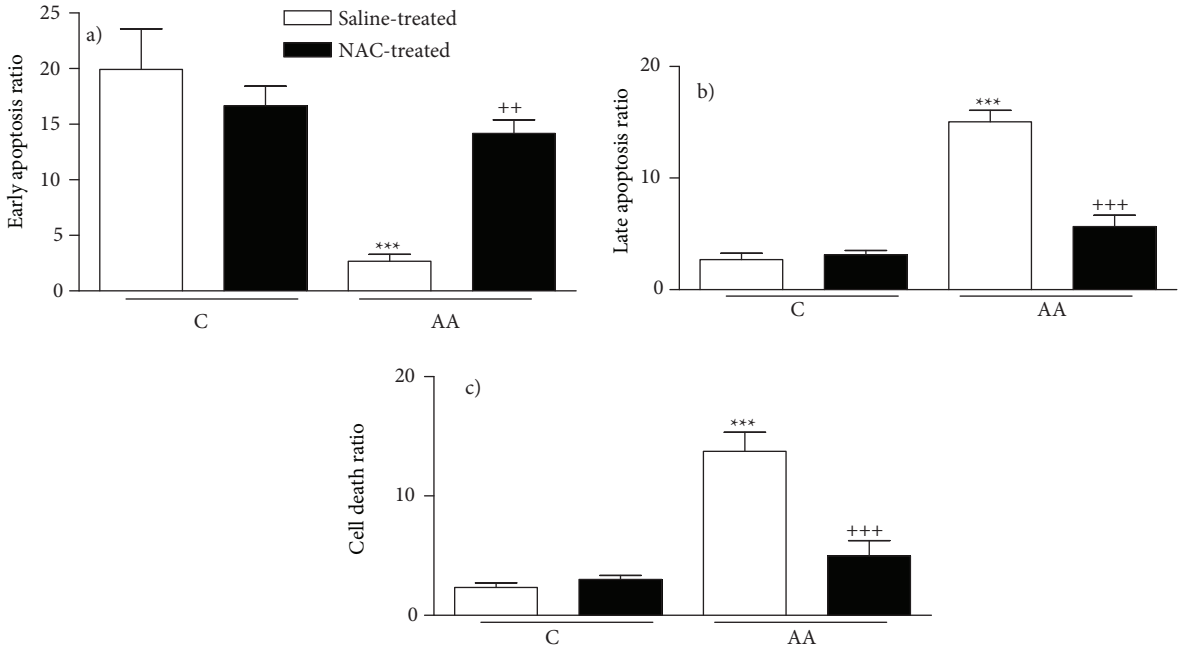

Figure 2. a) Early apoptosis, b) late apoptosis, and c) cell death in neutrophils in the blood samples of the saline- or NAC-treated control and AA groups. Apoptosis and cell death ratios were calculated by dividing the values after stimulation to the values obtained prior to PMA stimulation. ***P < 0.001 compared to the saline-treated control group; +++P < 0.001, compared to the saline-treated AA group.

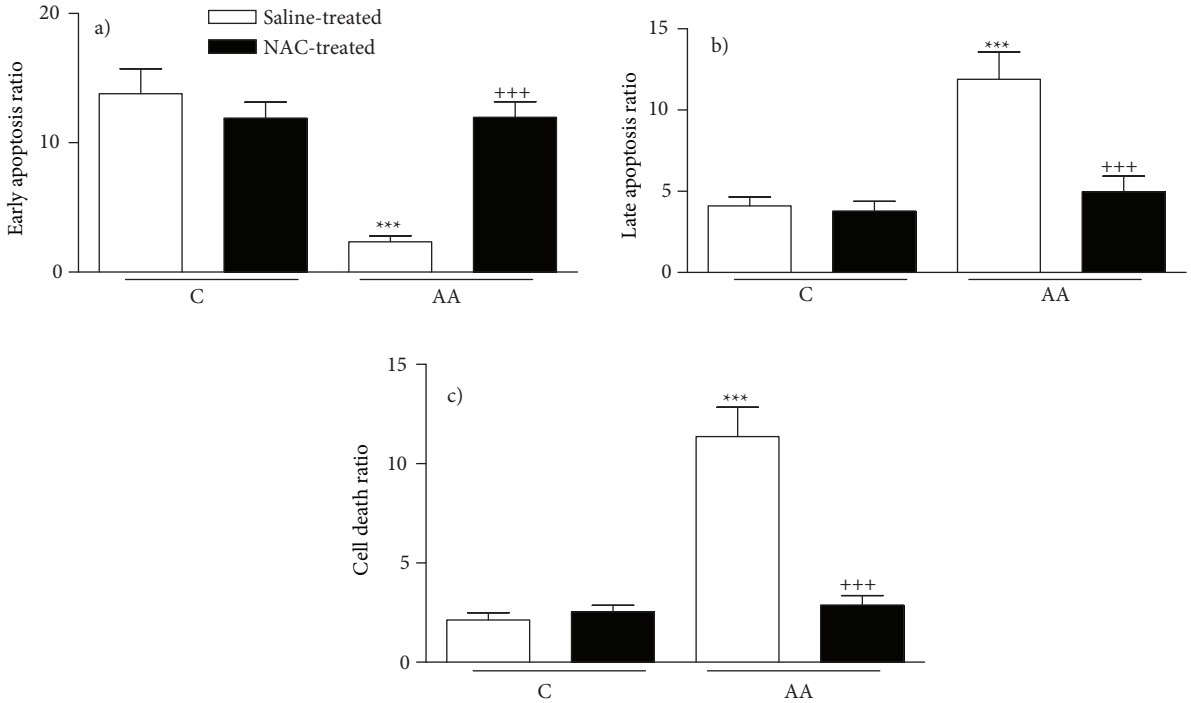

Figure 3. a) Early apoptosis, b) late apoptosis, and c) cell death in lymphocytes in the blood samples of the saline- or NAC-treated control and AA groups. Apoptosis and cell death ratios were calculated by dividing the values after stimulation to the values obtained prior to PMA stimulation. ***P < 0.001 compared to the saline-treated control group; +++P < 0.001, compared to the saline-treated AA group.

following the administration of AA (21). AA is capable of interacting with vital cellular nucelophiles possessing -SH, -NH2 or -OH. Therefore, it reacts with GSH in a similar manner and forms glutathione S-conjugates, which is the initial step in the biotransformation of electrophiles into mercapturic acids (22). In the present study, decreased GSH content and increased lipid peroxidation in tissues can be explained by the reaction of AA with GSH, which in turn causes the depletion of GSH and the enhancement of lipid peroxidation.

Moreover, the depletion of cellular GSH stores and the change in the redox status of the cell, in turn, may modulate gene expression directly or via the transcription factors that are redox-regulated, and may lead to apoptosis, cell proliferation, or transformation (23). This may explain significantly increased leukocyte apoptosis following AA administration in the present study. Furthermore, MPO, which is an endogenous lysosomal enzyme that removes H_2O_2 and catalyzes the formation of toxic hypochlorous acid, was increased significantly due to AA toxicity. Hypochlorous acid interacts with other small molecules to produce various ROS including hydroxyl radicals, singlet oxygen, peroxynitrite, and ozone (24). Previous studies have demonstrated that MPO derived chlorinated oxidants and hydroxyl radicals play key roles in inducing oxidative stress-mediated apoptosis in myeloid leukemia cells, with ROS being responsible for triggering apoptosis (25).

NAC is a precursor of GSH and it is used clinically for a broad spectrum of indications including mucolysis, detoxification after acetaminophen poisoning, respiratory distress syndrome, hyperoxia-induced pulmonary damage, HIV infection, cancer, and heart disease (26). However, it is still not clear if NAC is an effective antioxidant. In the present study, the antioxidant balance impaired by AA induction improved with NAC treatment in the tissues. Accordingly, increased proinflammatory cytokine expression can also be associated with the generation of free radicals, as the oxidative damage marker of DNA, 8-OHdG levels were significantly increased in the blood samples of the AA group. Moreover, MPO activities, which increased due to AA toxicity, reversed with NAC treatment, as NAC has been shown to work as a scavenger of the products of the MPO system (27). Collagen content, which increased in the tissues suggesting oxidative organ damage, was also reversed due to NAC treatment. This finding is consistent with the findings of Sato et al. (28), who showed that NAC substantially reduced the hydrogen peroxide induced elevation of cellular proliferation and collagen production.

In the literature, an inconsistency exists about the contribution of ROS to the regulation of apoptosis (29,30). In the present study, inducers of NF-κB such as TNF-α and IL-1 were increased significantly due to AA toxicity, which is associated with increased ROS. Moreover, leukocyte apoptosis, which increased significantly following AA administration, decreased with NAC treatment. Yang et al. (30) demonstrated that NAC inhibits NF-κB activation by reducing the inhibitor of kappa B kinase (IKK) activity, and this may in turn explain decreased leukocyte apoptosis following NAC treatment.

Most dry pet foods contain cereal grains or potatoes, and are processed at high temperatures and high pressure, making AA formation possible. On the other hand, the content and potential effects of AA formation in pet foods are unknown. To the best of our knowledge, this study is the first to investigate the effects of NAC in AA toxicity on the brain, lung, liver, kidney, and testes tissues of the rat by evaluating biochemical, inflammatory, oxidant-antioxidant, and apoptotic markers. Our results demonstrate that in AA toxicity NAC protected these tissues by inhibiting neutrophil infiltration, balancing the oxidant-antioxidant status, and regulating the generation of inflammatory mediators. Therefore, supplementing with NAC can be useful when there is a risk of AA toxicity.

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