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Effects of N-Acetyl Cysteine on the Expression of Matrix Metalloproteinases 2 and 9 in the Lung Tissue of Rats Exposed to Cadmium

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Abstract

Background: Cadmium (Cd) is a natural and heavy metal, which is widely widespread in the atmosphere. Studies report that environmental exposure to Cd increases the risk of various disorders, such as pulmonary diseases. On the other hand, Cd increases the reactive oxygen species (ROS), which interacts with biomolecules (e.g. DNA, proteins, and lipids) and causes severe damages. In addition, Cd may play a role in the dysregulation of the expression and activity of matrix metalloproteinases (MMPs). Since ROS and oxidative stress are likely the main reasons for MMPs dysregulation, antioxidants therapy may protect tissues against Cd-induced damages. Furthermore, N-acetylcysteine (NAC) protects cells against oxidative stress and toxic compounds.

Objectives: This study aimed to investigate the effect of cadmium (Cd) on the matrix metalloproteinases (*MMPs*) -2 and -9 expression in the lung, and the role of N-acetylcysteine (NAC) in preserving the lung cells against Cd toxicity.

Methods: The rats were randomly divided into five groups of G1 (control), G2 (single dose of Cd), G3 (continuous dose of Cd), G4 (single dose of Cd+NAC), and G5 (continuous dose of Cd+NAC). The level of Cd in the blood and lung tissue was measured by atomic absorption spectroscopy. Moreover, the expression of *MMP2* and *MMP9* genes was evaluated using RT-PCR.

Results: Single and continuous exposure to Cd caused a significant increase in serum and the lung tissue of Cd in G2 (0.23±0.04 mg/L and 0.35±0.047 µg/g tissue) and G3 (0.50±0.068 mg/L and 0.81±0.063 µg/g tissue) groups, compared to other groups (P<0.001). The NAC supplementation significantly decreased Cd levels in the serum and lung tissue samples of rats exposed to single or continuous Cd (P<0.001). Furthermore, exposure to a single and continuous dose of Cd caused a significant increase in the *MMP2* expression by 3.24-fold (P=0.003) and 11.9-fold (P<0.001), respectively. Additionally, treatment with single and continuous dose treatment of Cd led to a significant increase in the *MMP9* expression by 3.20-fold (P=0.004) and 7.54-fold (P<0.001), respectively. The NAC treatments decreased the expression of *MMP2* and *MMP9* in the lung of rats exposed to a single or continuous dose of Cd.

Conclusion: The Cd exposure was strongly associated with the accumulation of Cd and overexpression of *MMP2* and *MMP9* in the lung tissue. Moreover, the NAC can protect the lungs against Cd toxicity by decreasing Cd and down-regulating *MMP*s.

Keywords: Cadmium, *MMP*2, *MMP*9, N-Acetyl cysteine, Rat

1. Background

Cadmium (Cd) is a natural and heavy metal which is widespread widely in the atmosphere (1). Since this toxic metal can be accumulated in our surrounding area, it has become a major health problem throughout the world (2). Studies report that environmental exposure to Cd increases the risk of various disorders, such as pulmonary diseases, kidney and diabetic nephropathy, cardiovascular disease, osteoporosis, lung and hepatic cancers, neurotoxicity, and diabetes (3-8). However, the exact cellular and molecular mechanisms in which Cd induces these abnormalities are not well-understood. According to studies, cellular toxicity is induced by Cd using multiple mechanisms, especially by inhibiting enzymes that are involved in DNA synthesis, cellular energy metabolism, and inhibition of DNA repair (9,10). Similarly, reactive oxygen species (ROS) induce oxidative stress that is proposed as the main mechanism of Cd toxicity (10,11). The ROS interacts with biomolecules (e.g. DNA, proteins, and lipids) and causes severe damages, especially DNA breaks or mutations, followed by cell death and apoptosis (12,13).

Studies showed that Cd exposure can be associated with the overproduction of ROSs, such as hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\bullet}$), and hydroxyl radicals ($OH^{\bullet}$) (14). According to previous studies, chronic exposure to Cd can be associated with the overproduction of ROS (15), lipid peroxidation (16), and antioxidant depletion (17,18). Moreover, it is shown to correlate with an increase in proinflammatory cytokines, including interleukin-6 (IL-6) and IL-8 (19). Dysregulation of matrix metalloproteinases (*MMP*s) expression or their activity is another possible mechanism of chronic exposure to Cd (20,). Matrix metalloproteinases are a

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group of zinc-dependent endopeptidase enzymes that are involved in the extracellular matrix (ECM) protein degradation (21). These enzymes have a wide range of physiological functions, including embryogenesis, wound healing, bone growth, as well as regeneration growth, migration, differentiation, inflammatory processes, and apoptosis (22, 23). However, *MMPs* may cause pathological conditions in case their expression pattern is dysregulated (24). There are conflicting results regarding the effect of Cd on *MMPs* expression or their activity. Fomenko et al. (20) demonstrated that Cd exposure decreased the activity of *MMP*2 but increased the activity of pro*MMP9* in the brain of rats. In another study, Lacorte et al. (25) reported that Cd exposure deregulated the balance in the extracellular matrix turnover through *MMPs* downregulation.

Since ROS and oxidative stress are probably the main reasons for *MMPs* dysregulation, antioxidant therapy may protect tissues against Cd-induced damages. Therefore, it is believed that antioxidant therapy can be helpful to reduce the toxicity of Cd in exposed individuals. N-acetylcysteine (NAC) as an acetylated cysteine residue protects cells against oxidative stress and toxic compounds (26, 27). Recent studies have demonstrated that NAC can decline heavy metal-induced hepatotoxicity (28), renal toxicity, kidney and brain (29) reproductive toxicity (30), DNA damage (31), oxidative stress, inflammation, and apoptosis (32). Nevertheless, little research has been conducted on the protective effects of NAC during and after Cd treatment on the lung tissue. Since the lung seems to be the main target for Cd toxicity, it is hypothesized that NAC supplementation may help maintain lung health by excreting toxic metabolites, improving antioxidants, as well as declining oxidative stress, and consequently *MMPs* expression.

2. Objectives

This study aimed to investigate the protective effect of NAC supplementation on histological alterations, contents of Cd, as well as the expression of *MMP*2 and *MMP*9 in rats exposed to Cd.

3. Methods

3.1. Animals

In total, 30 male Wistar rats with 8-10 weeks of age and bodyweight of 150-200 g were bought from the laboratory of the animal research center at Pasteur Institute of Iran (Tehran, Iran). All rats were adapted to the lab environment for one week and then randomly divided into five groups of G1 (control), G2 (single dose of Cd), G3 (continuous dose of Cd), G4 (single dose of Cd+NAC), and G5 (continuous dose of Cd+NAC). The rats per group were housed three in each cage (30×15×15 cm) in a standard climate room (at the temperature and humidity of 22±2°C and 50%±5%, respectively, as well as a 12:12 light/dark cycle); moreover, they had free access to food (10g/kg/day) and tap water.

3.2. Treatments

Normal pellet and water were utilized to feed the rats in the G1 group (control) for four weeks, and the animals in the G2 group received a single gavage of Cd solution (80 mg/kg) on the first day of examination. On the other hand, the animals in the G3 group received a continuous gavage of Cd solution (2.5 mg/kg) every other day for four weeks. Furthermore, the rats in the G4 group received a combination of Cd (80 mg/kg) and NAC (50 mg/kg) solutions at the same time on the first day of the examination, and the animals in the G5 group gained a continuous administration of Cd (2.5 mg/kg) and NAC (50 mg/kg) solutions every other day for four weeks.

3.3. Tissue and blood sample collection

Xylazine (3-5 mg/kg) and ketamine (30-50 mg/kg) were utilized 48 h after the final treatment to anesthetize the rats. Subsequently, blood samples were obtained from the abdominal aorta to assess the serum Cd contents. For the histological study, lung tissues were removed and fixed in 10% formalin for at least 48 h. Fragments were dehydrated in the graded series of ethanol, embedded in paraffin, and sectioned using an automatic microtome at 4-5 µm thickness. The sectioned tissues were stained with hematoxylin-eosin and evaluated for morphological and histological parameters by light microscope. A fragment of lung tissue (~50-100 mg) was separated and homogenized in phosphate buffer (with pH 7.0) at 4°C with a homogenizer (Hielscher, UP100H). The homogenized tissue was centrifuged at 12000 rpm/4°C for 15 min (33). The supernatants were then collected and stored at -80°C for further analysis.

3.4. Measurement of Cadmium

Considering the Cd analyses, lung tissues (~100 mg) were dried overnight at 75°C and then digested in approximately 10× of the dry tissue mass of nitric acid. The digested samples were diluted 5-fold by deionized water. For the analysis of Cd in serum, blood samples were centrifuged at 600×g for 10 min. After centrifugation, supernatants were diluted 5-fold by deionized water. Eventually, the level of serum and lung tissue Cd was measured by atomic absorption spectroscopy (AAS; Perkin Elmer model 2380). Different concentrations of Cd (0.01-0.8 mg/L) and concentrations of Cd (0.01-0.8 µg) were used to plot the standard curve for serum and tissue Cd analysis, respectively.

3.5. Gene expression analysis

The RNX-Plus (SinaClon; RN7713C) Kit was applied for the extraction of total RNA from homogenized lung tissues. In addition, the quantity and quality of extracted RNAs were considered using a Nanodrop ND-1000 spectrophotometer (Thermo Sci., Newington, NH). Furthermore, electrophoresis on 1% agarose gel was performed to consider the quality of extracted RNAs. Afterward, cDNA was synthesized using Revert Aid Reverse Transcriptase Enzyme (Thermo science, Germ any) and random hexamer primers (Thermo science, Germany) at 42°C for 1 h.

A Rotor-Gene 6000 (Corbett Research, Australia) thermocycler was also applied in 40 cycles for amplification. Each reaction had 5 µl master mix and 100 nM primers. Primer sequences were *MMP2*, 5/-CCGTCGCCCATCATCAAGT-3/ (forward), 5/-GCAGCCATAGAAAGTGTTCAGGT-3/ (reverse); *MMP9*, 5/-ACCACCGCCAACTATGACCAG-3/ (forward), 5/-TGCTTGCCCAGGAAGACGA-3/ (reverse); glyceral dehyde 3-phosphate dehydrogenase (GAPDH), 5/-AAGTTCAACGGCACAGTCAAGG-3/ (forward); and 5/-CATACTCAGCACCAGCATCACC-3/ (reverse). The levels of mRNA were normalized relative to the amount of GAPDH mRNA, and the relative expression of the studied genes was calculated using the $2^{-\Delta\Delta Ct}$ method.

3.6. Statistical Analysis

The data were analyzed using SPSS software (version 19) and presented as mean±SD through one-way ANOVA and the post-hoc Tukey test to compare the mean of all data among the groups. A *p*-value less than 0.05 was considered statistically significant.

4. Results

Histopathological examination of the lung tissue in each group revealed no abnormalities in the control group (G1), while the sections of lung tissue from rats in the G3 group showed an elevated inflammation. Moreover, the sections of the lung from rats treated with NAC (G4 and G5) showed milder abnormalities, compared to the rats in G2 and G3 groups. Combined therapy with NAC declined the number of inflammatory cells along with mild inflammation in the lung of rats exposed to Cd (Figure 1).

A significant difference was observed among the groups in terms of the expression pattern of *MMP2* and *MMP9* genes ($P<0.001$). Overall, continuous or single-dose treatment of Cd significantly increased the expression of *MMP2* and *MMP9* in the exposed rats, while NAC supplementation significantly decreased the expression of these genes(Figure 2). Compared to the control group, single and continuous dose treatment of Cd caused a significant increase in *MMP2* expression by 3.24-fold ($P=0.003$) and 11.9-fold ($P<0.001$), respectively (Table 1). Moreover, single and continuous dose treatment of Cd led to a significant increase in *MMP9* expression by 3.20-fold ($P=0.004$) and 7.54-fold ($P<0.001$), respectively (Table 2).

In contrast, the rats that received a combination of NAC and Cd showed a significant decrease in *MMPs* expression, compared to the animals treated with single or continuous Cd alone. Compared to rats treated with a single dose of Cd, *MMP2* and *MMP9* expression in animals received single Cd + NAC was significantly decreased by 2.68-fold ($P=0.006$) and 1.81-fold ($P=0.039$), respectively (Tables 1 and 2).

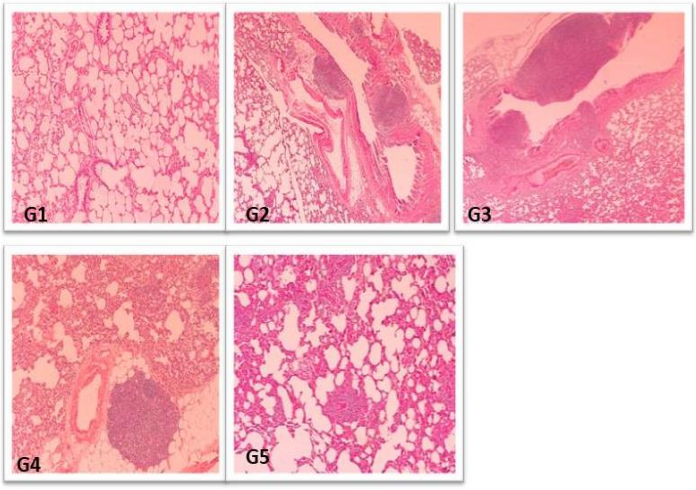

Figure 1. Sections of the lung tissue from different groups. The lungs of rats in the control group (G1) were normal in structure, while the sections from rats in the continuous group (G2 and G3) showed increased blood in the central vein and elevated inflammatory cells. Combined therapy with NAC declined the number of inflammatory cells along with mild inflammation in Cd exposed groups (G4 and G5) (X20 magnification).

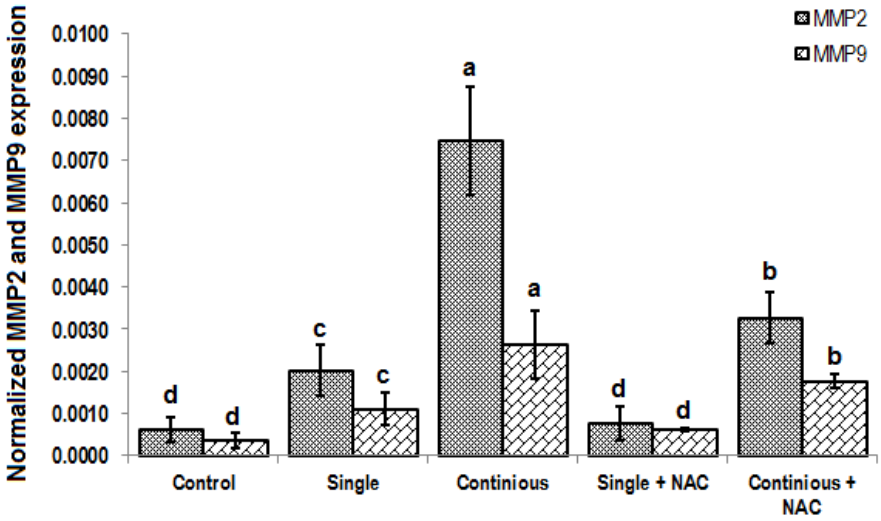

Figure 2. Comparison of the mean values of mRNA levels of MMP2 and MMP9. Gene expression was detected by Real-Time PCR. There was no significant difference among the groups with similar symbols in terms of the mRNA levels of MMP2 and MMP9. The mean mRNA level of MMP2 and MMP9 was in the order of a>b>c>d. One-way ANOVA: Post-hoc Tukey test was applied to compare the mean values of MMP2 and MMP9 expression pattern among all groups.

Furthermore, compared to rats exposed to a continuous dose of Cd, *MMP2* and *MMP9* expression in animals received continuous Cd + NAC was significantly decreased by 2.28-fold (P<0.001) and 1.49-fold (P=0.046), respectively (Tables 1 and 2).

The mean of Cd concentrations in the blood and lung tissue of rats exposed to single Cd were 0.23±0.04 mg/L and 0.35±0.047 µg/g, respectively. However, the mean values of Cd contents in the blood and lung tissues of rats exposed to continuous Cd were 0.50±0.068 mg/L and 0.81±0.063 µg/g, respectively (Figures 3 and 4). The NAC supplementation significantly decreased Cd concentrations in serum and tissue samples of rats exposed to single or continuous Cd. The mean concentration of Cd in the lung and serum of the

Table 1. Comparison of the fold change ratio of the *MMP2* expression

	Fold-change ratio	Up-/down-regulation	P-value
Single vs. control	3.24	Up-regulated	0.003
Continuous vs. control	11.9	Up-regulated	<0.001
NAC+single vs. control	1.21	Up-regulated	0.75
NAC+continuous vs. control	5.22	Up-regulated	<0.001
Continuous vs. Single	3.68	Up-regulated	<0.001
Single vs. NAC+continuous	1.61	Down-regulated	0.007
Single+NAC vs. single	2.68	Down-regulated	0.006
Single+NAC vs. continuous	3.68	Down-regulated	<0.001
Single+NAC vs. NAC+continuous	4.31	Down-regulated	<0.001
Continuous+NAC vs. continuous	2.28	Down-regulated	<0.001

*P<0.05 is considered statistically significant; One-way ANOVA: Post-hoc Tukey test was applied to compare the mean values of *MMP2* expression pattern among all groups.

Table 2. Comparison of the fold change ratio of the *MMP9* expression

	Fold-change ratio	Up-/down-regulation	p-value
Single vs. control	3.20	Up-regulated	0.004
Continuous vs. control	7.54	Up-regulated	<0.001
NAC+single vs. control	1.77	Up-regulated	0.27
NAC+continuous vs. control	5.06	Up-regulated	<0.001
Continuous vs. Single	2.36	Up-regulated	<0.001
Single vs. NAC+continuous	1.58	Down-regulated	0.013
Single+NAC vs. single	1.81	Down-regulated	0.049
Single+NAC vs. continuous	2.36	Down-regulated	<0.001
Single+NAC vs. NAC+continuous	2.86	Down-regulated	<0.001
Continuous+NAC vs. continuous	1.49	Down-regulated	0.046

*P<0.05 is considered statistically significant; One-way ANOVA: Post-hoc Tukey test was applied to compare the mean values of *MMP9* expression pattern among all groups.

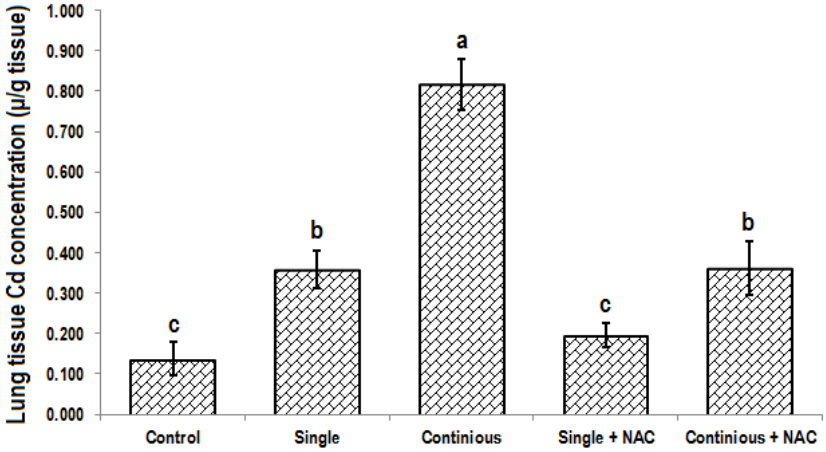

Figure 3. Comparison of the mean of the Cd levels in the lung tissue of rats in different groups. The mean of Cd level was in the order of a>b>c. One-Way ANOVA: Post-hoc Tukey test was applied to compare the mean values of Cd among all groups.

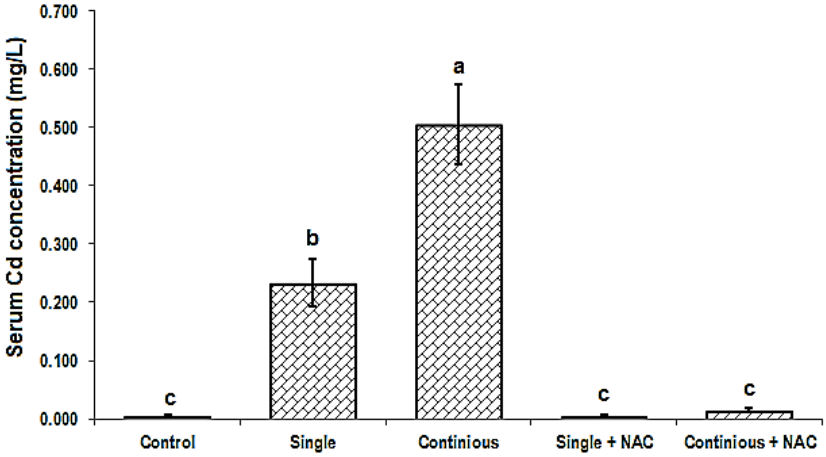

Figure 4. Comparison of the mean of Cd levels in the serum of rats in different groups. The mean of Cd level was in the order of a>b>c. One-Way ANOVA: Post-hoc Tukey test was utilized to compare the mean values of Cd among all groups.

rats treated with NAC+single Cd (0.19±0.029 µg/g tissue and 0.006±0.001 mg/L, respectively) was relatively similar to that in control. Although NAC supplementation significantly decreased lung tissue and serum Cd levels (0.36±0.066 µg/g tissue and 0.015±0.003 mg/L, respectively)in the G5 group, Cd levels were still greater than those in the G1 (control) group (Figure 3).

5. Discussion

This study evaluated the effect of NAC supplementation on histological changes, as well as *MMP2* and *MMP9* gene expression in the lung tissue of rats exposed to single or continuous dose treatment of Cd. The data have revealed that Cd exposure, especially continuous exposure, is significantly associated with increased mean levels of lung and blood Cd in the exposed animals. The data implicate that the accumulation of Cd in the lung tissue is the main mechanism of its toxicity during the chronic phase of injury. It was also found that Cd administration, especially chronic exposure, significantly resulted in the overexpression of *MMP2* and *MMP9* genes in the lung tissues of the exposed rats.

The findings support the idea that the toxicological effect of Cd on the lung tissue is probably mediated via the overexpression of these proteinases. In a study, Yaghooti et al. (34) found that Cd exposure significantly led to *MMP9* expression in lung cells in a dose-dependent manner which was in agreement with the findings of the present study. However, the exact mechanism in which Cd increases the expression of *MMP2* and *MMP9* is not well-understood. It appears that oxidative stress and inflammation are the main indirect mechanism of Cd toxicity on the overexpression of these *MMPs*. To support this hypothesis, several experimental studies illustrated that Cd exposure decreased the mean level

of enzymatic and non-enzymatic antioxidants in different tissues.

Prozialeck et al. (35) reported that Cd treatment caused a significant decrease in the renal cortical Zn level. Previous studies also indicated that the activation of resident macrophages of tissues in response to Cd was an important source for inflammatory mediators, such as IL-1, IL-6, TNF-α, and IL-8 which are the mediators of *MMPs* expression and activation (36,37). According to a study conducted by Fomenko et al. (20), Cd in a higher dose caused a decrease in the *MMP2* activity but increased that of pro*MMP9* in the brain.

Antioxidant therapy can be helpful to decrease the pathological effects of Cd exposure due to the fact that Cd exposure is correlated with inflammatory responses, oxidative stress, and antioxidant depletion. In this study, the protective effect of NAC was considered on the level of Cd, as well as the expression of *MMP2* and *MMP9* in the lung of rats exposed to Cd. Furthermore, Cd exposure not only increased the *MMP2* and *MMP9* expression but also induced lung tissue lesions and structural abnormalities. These findings revealed that the animals suffered from inflammation and proteolytic activity of *MMP2* and *MMP9* after long-term exposure to Cd. Interestingly, NAC supplementation significantly decreased the mean level of Cd and expression of *MMP2* and *MMP9*. According to these results, NAC has a significant effect on the protection of the lung cells against toxicity induced by Cd.

In this regard, several studies have indicated that NAC has important roles in the prevention of harmful effects of different heavy metals on various tissues, including the kidney, liver, and brain (28,38-40). According to a study conducted by Da Silva et al., the co-administration of the NAC could enhance the detrimental effects of arsenic on the genital system of the males (1). Reddy et al. found that the intraperitoneal injection of the NAC led to an increase in the weights of reproductive organs, reduction in oxidative stress induced by arsenic, and improvement in steroidogenesis among the mice exposed to arsenic. This indicated the remarkable effect of NAC on counteracting the oxidative stress induced by arsenic and restoring the reproduction which was suppressed in male mice (30).

The anti-apoptotic effect of NAC was also considered in multiple studies (41,42). Sanker et al. showed that heavy metals, such as arsenic, induced lipid peroxidation and glutathione depletion, followed by a decrease in the activities of enzymes, such as superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase. They also demonstrated that these metals increased the activity of serum alanine and aspartate amino-transferase and caused histological alterations in the liver indicating hepatotoxicity (43). The results of the aforementioned study revealed that antioxidants, including curcumin treatment (100 mg/kg), could reduce all these effects. These findings demonstrate that antioxidant therapy can protect different organs against Cd toxicity by a decrease in DNA damage, lipid peroxidation, and ROS generation along with an increase in the antioxidant activity level (31). Therefore, according to previous accomplished data and our findings, the overexpression of *MMP2* and *MMP9* is the main reason for Cd cytotoxicity in the lung tissue. On the other hand, NAC diminishes the genotoxicity and cytotoxicity effects of Cd in the lung.

6. Conclusion

In conclusion, the findings of the current study revealed that Cd exposure, especially chronic exposure to Cd, is strongly associated with the accumulation of Cd in the lung and overexpression of *MMP2* and *MMP9* in the lung tissue. The NAC can help protect the lung tissue against Cd toxicity by Cd scavenging and down-regulation of *MMP2* and *MMP9*.

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Footnotes

Authors' Contribution: All the authors had an effective contribution to the manuscript preparation.
Conflict of Interests: The authors declare that they have no conflict of interest.
Ethical Approval: The study protocol was approved by the Research Ethics Committee of Rasht Branch, Islamic Azad University, Rasht, Iran (ID: IR.IAU. RASHT.REC.1398.054).
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Informed consent: Not applicable

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