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Original Article

Protective Effects of Bromelain against Cadmium-Induced Pulmonary Intoxication in Rats: A Histopathologic and Cytologic Study

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Abstract

Bromelain is the active substance of pineapple with a variety of therapeutic properties. In this study, the possible protective effects of bromelain were assessed against cadmium acute intratracheal exposure and its bronchopulmonary cytologic and histopathologic consequences. For this purpose, the following treatments were performed on 11 groups of Wistar rats: group 1 was negative control; groups2 and 3 received Cadmium Chloride ($CdCl_2$) 400 µg/rat intratracheally and sampled after 5 and 10 days, respectively; groups4 and 5received bromelain 20 mg/kg orally (PO) from 14 days before until 5 and 10 days after $CdCl_2$ instillation, respectively; groups6 and 7received bromelain 40 mg/kg from 14 days before until 5 and 10 days after $CdCl_2$ instillation, respectively; group 8received bromelain 40 mg/kg for 24 days; groups9 and 10: celecoxib 25 mg/kg PO from 1day before until 5 and 10 days after $CdCl_2$ instillation, respectively; group 11 received celecoxib for 11 days. Cytologic evaluation of bronchoalveolar lavage fluid revealed that intratracheal cadmium administration resulted in a significant rise in total cell count, epithelial cells, neutrophils, and eosinophils, 5- and 10-days post-exposure. Treatment with bromelain either in low or high doses in cadmium-exposed rats resulted in a significant reduction of neutrophil count. Bromelain treatment could not completely prevent or recover interstitial pneumonia and fibrinous bronchopneumonia in cadmium exposed rats. However, administration of low doses resulted in a significant decrease of semi quantitative histopathologic scores, including pneumonia and cellular infiltration indices. In conclusion, bromelain may help to improve the cytological and histopathological complications following cadmium intoxication in the lungs.

Keywords: Bromelain, Bronchoalveolar lavage fluid, Cadmium, Pneumonia, Pulmonary intoxication

1. Introduction

Cadmium is a heavy metal that is considered to be a job-related and environmental toxicant with a very long half-life *in vivo* (1). Cadmium can cause severe damage to several organs, including nephrotoxicity, hepatotoxicity, and pneumotoxicity (1). Occupational exposure to airborne cadmium may take place by welding, smelting, automobile emissions, manufacturing of electric equipment, alloys, and pigments (2). Inhalation of a high concentration of cadmium fumes (e.g. in welding accidents) can cause acute pulmonary damage, pneumonitis, pulmonary emphysema, and altered surfactant production resulting in prolonged impairment of pulmonary function (2).

Although it is not yet clear how cadmium causes toxicity, many studies suggest that it might interfere with essential metals, generate oxidative stress, inhibit DNA restoration, affect apoptosis, and cause

respiratory system inflammation (3). Many research findings suggest cadmium to have pro-inflammatory properties that can up-regulate the inflammation mediators (4). Hence, the administration of drugs/agents with anti-inflammatory characteristics might be beneficial in preventing or reducing cadmium-induced organ damage.

Bromelain is the active substance of pineapple which is a combination of protease enzymes. It has been shown to have anti-inflammation properties and act as an immunomodulator, wound healer, and circulatory enhancer which leads to extensive administration of bromelain. In addition, as herbal medication, its global approval is in part owing to a background of its nontoxic application and rare side effects (5). However, bromelain was not evaluated as a protecting agent in cadmium-generated lung toxicity.

Celecoxib is a non-steroidal anti-inflammatory drug (NSAID) with analgesic and antipyretic properties. Celecoxib belongs to a group of NSAIDs that are considered selective cyclooxygenase-2 (COX-2) inhibitors commonly known as coxibs (6).Results of several studies have shown that bromelain also exerts its anti-inflammatory effects by inhibiting COX-2 expression (7). Therefore, in this study, the two drugs were compared to clarify the mechanism of the possible effects of bromelain on cadmium-induced inflammation.

Hence, this study was performed to investigate the possible protective impacts of bromelain in comparison to celecoxib, as an anti-inflammatory drug, against acute intratracheal cadmium contact and its bronchopulmonary cytologic and histopathologic consequences.

2. Materials and Methods

2.1. Laboratory Animals

In total, 66 albino male Wistar rats within the weight range of 250-300 g were kept in a room at the temperature and humidity of 24±2 ˚C and 55±5%, respectively, with a 12 h light/12 h dark cycle. The rats were fed a commercial laboratory pellet diet and tap water *ad libitum*.

2.2. Experimental Design

The study was performed on 11 equal groups (6 rats in each group) that were treated as follows:

Group 1 (control): received 400 µl of normal saline intratracheally (IT) and were sampled after 10 days.

Group 2: received 400 µg/rat Cadmium Chloride ($CdCl_2$) IT and were sampled after 5 days (8).

Group 3: received 400 µg/rat $CdCl_2$ IT and were sampled after 10 days.

Group 4: received 400 µg/rat $CdCl_2$ IT and 20 mg/kg bromelain (9) PO by gavage daily from 14 days prior to cadmium instillation until 5 days after it.

Group 5: received 400 µg/rat $CdCl_2$ IT and 20 mg/kg bromelain PO daily from 14 days prior to cadmium instillation until 10 days after it.

Group 6: received 400 µg/rat $CdCl_2$ IT and 40 mg/kg bromelain (10) PO daily from 14 days prior to cadmium instillation until 5 days after it.

Group 7:received 400 µg/rat $CdCl_2$ IT and 40 mg/kg bromelain PO daily from 14 days prior to cadmium instillation until 10 days after it.

Group 8: received 40 mg/kg bromelain PO daily for 24 days.

Group 9: received 400 µg/rat $CdCl_2$ IT and 25 mg/kg celecoxib (11) PO daily from 1 day prior to cadmium instillation until 5 days after it.

Group 10: received 400 µg/rat $CdCl_2$ IT and 25 mg/kg celecoxib PO daily from 1day prior to cadmium instillation until 10 days after it.

Group 11: received 25 mg/kg celecoxib PO daily for 11 days.

2.3. Intratracheal Injection Method

After anesthesia by administration of ketamine (60 mg/kg/IP) plus xylazine (5 mg/kg/IP), the rats were placed on a slant wooden board with an angle of 60 degrees. Accordingly, their backs were against the board and they were suspended from their incisors on a wire. Their tongues were softly pulled out and held to the side of the oral cavity with blunt forceps. The

syringe containing the inoculum (400 µl/rat) was attached to a curved gavage needle and the needle was inserted into the pharynx. The plunger was pushed evenly to deliver the inoculum and the needle was pulled out of the pharynx as soon as possible. The nostrils were blocked by fingers, and the tongue restriction was sustained up to a minimum of two deep breaths. The rats were held upright for a few seconds to allow inoculum to be inhaled into the lung.

2.4. Bronchoalveolar Lavage

The euthanized rats were placed in the dorsal position and incised at midline from the mandible to the abdominal cavity. Afterward, the thorax was opened and the right bronchus was ligated with nylon suture to prevent penetration of lavage fluid into the right lung and preserve it for subsequent histopathologic assessment. A minor incision was made in the trachea about one inch above the branching where a catheter was inserted and the bronchoalveolar lavage was performed with 2.5 ml saline two sequential times. The aspirated fluids were merged and held on ice.

The bronchoalveolar lavage fluid (BALF) samples were assessed for total cell counts using an automatic cell counter (Mindray 2800-vet, China). The samples were subsequently centrifuged at 300×g for 10 min and the pellet was suspended in 0.25 ml fetal bovine serum. This suspension was used to prepare two microscope slides, which were fixed with methanol (Merck, Germany) and stained using Giemsa solution (Baharafshan, Iran). The slides from each treated rat were microscopically examined for morphologic differential cell counts of leukocytes, epithelial cells, and alveolar macrophages.

2.5. Histopathologic Assessment

After collection of BALF, the right lung and its accompanying bronchus were excised. The tissues were then stored in 10% formalin fixative and the fixative was replaced after 24 h for better fixation. Afterward, paraffin embedding and tissue sectioning were performed followed by staining with Hematoxylin and Eosin for microscopic observation. The histopathologic examination was performed both qualitatively and semi-quantitatively.

2.6. Semiquantitative Hstopathologic Scoring Method

Two independent histopathologic patterns were evaluated in this study. Firstly, cell infiltration surrounds pulmonary arteries, veins, and bronchioles. Secondly, acute pulmonary inflammation involves both the interstitial spaces and alveoli. Each pattern was semi-quantitatively scored. The severity grade was multiplied by the extent of involvement to generate apathology index (PI) (range: 0 - 9). Eventually, the pneumonitis and periluminal infiltrate PIs were added together to determine the total index (range:0-18) (12).

2.7. Statistical Analysis

The data were statistically analyzed in SPSS software (version 16, SPSS Inc., Chicago, IL, USA). The results were stated as mean±standard error. Nonparametric Kruskal-Wallis test was used for the analysis of histopathologic and cytological data. It should be mentioned that a p-value of less than 0.05 represented statistical significance.

3. Results

3.1. Bronchoalveolar Lavage Fluid Cytology Results

Based on the comparison of total and differential cell counts in the BALF revealed that the highest values of total cells, epithelial cells, neutrophils, and eosinophils were observed in group 2 (Cadmium exposure, sampled at day 5). Moreover, it was found that the maximum macrophage count was observed in group 3 (Cadmium exposure, sampled at day 10) (Figure 1). Low dose bromelain treatment for 24 days in cadmium-exposed rats (group 5) resulted in the reduction of the total count and various cell types. However, this alteration was not statistically significant except in neutrophil count ($P<0.05$).

High-dose bromelain administration in cadmium-exposed rats (groups 6 and 7) could not induce any significant decrease in total and differential counts,

except in neutrophils, compared to group 2 (P<0.05). Rats in group 8 (high dose bromelain treatment for 24 days) did not show any significant difference in terms of BALF total cells and various cell types, compared to the control group, except lymphocyte count which underwent a significant increase (P<0.05). No significant change in total or differential cell counts were noted in celecoxib treatment with and without cadmium, compared to the control group (P>0.05).

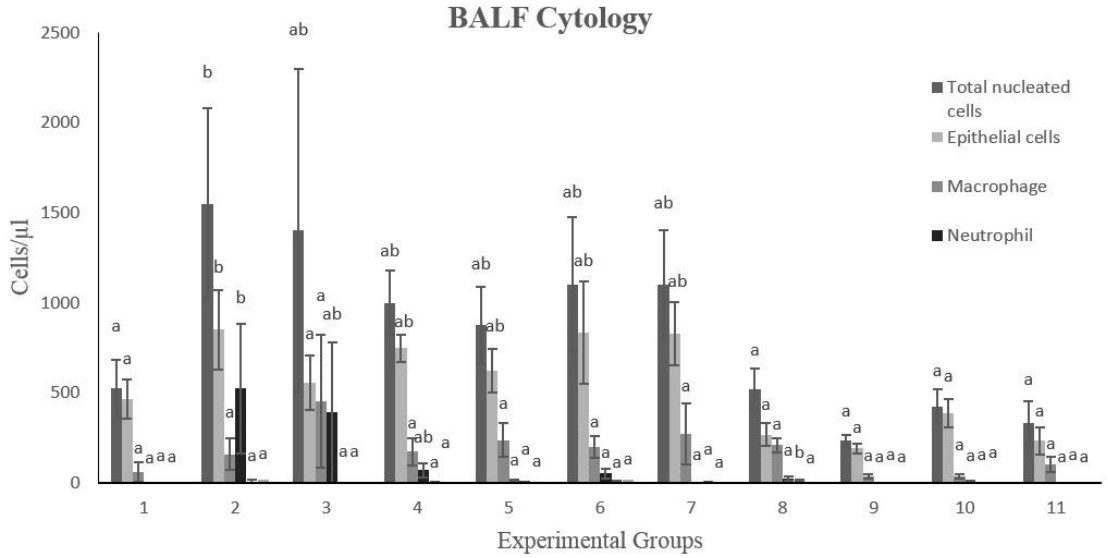

Figure 1. Cyologic results of bronchoalveolar lavage fluid (BALF) in different groups as mean±standard error. Study groups: 1) Control; 2 and 3) $CdCl_2$, 5 and 10d, respectively; 4 and 5) Bromelain 20+$CdCl_2$, 5 and 10d, respectively; 6 and 7) Bromelain 40+$CdCl_2$, 5 and 10d, respectively; 8) Bromelain 40, 24 d; 9 and 10) Celecoxib+$CdCl_2$, 5 and 10d, respectively; 11) Celecoxib, 11d. Different lowercase letters above columns in each data series represent statistically significant differences between groups.

3.2. Qualitative Histopathology Results

The microscopic examination of the lungs of all rats in group 2 (cadmium exposure, sampled at day 5) revealed severe interstitial pneumonia (Figure 2A) along with hemorrhage. In these cases, there was an increase in the interstitial pulmonary tissue thickness. These effects were due to the infiltration of inflammatory cells, especially neutrophils and hyperemia (Figure 2B).

Fibrinous bronchopneumonia was also diagnosed in two cases. In these cases, pink exudates containing fibrin strands were observed inside the alveoli and around the vessels (Figures 2C and 2D). A high number of inflammatory cells, especially neutrophils, were found around the airways and vessels, inside the alveoli, bronchioles, and interstitial tissues. In addition, the accumulation of necrotic cells in the interstitial pulmonary tissue was observed in some areas (Figure 2E). Moreover, necrosis and exfoliation of epithelial cells of bronchi and bronchioles occurred as well (Figure 2F).

Hyperemia and hemorrhage were other microscopic features. In groups 3 (cadmium exposure, sampled at day 10) and 4 (low dose bromelain treatment for 19 days plus cadmium exposure), all rats showed severe interstitial pneumonia. Fibrinous bronchopneumonia was also observed in one rat in each of the above-mentioned groups. Low-dose bromelain treatment for 24 days in cadmium-exposed rats (groups 5) resulted in moderate interstitial pneumonia (Figure 3A and 3B) plus fibrinous bronchopneumonia in one case.

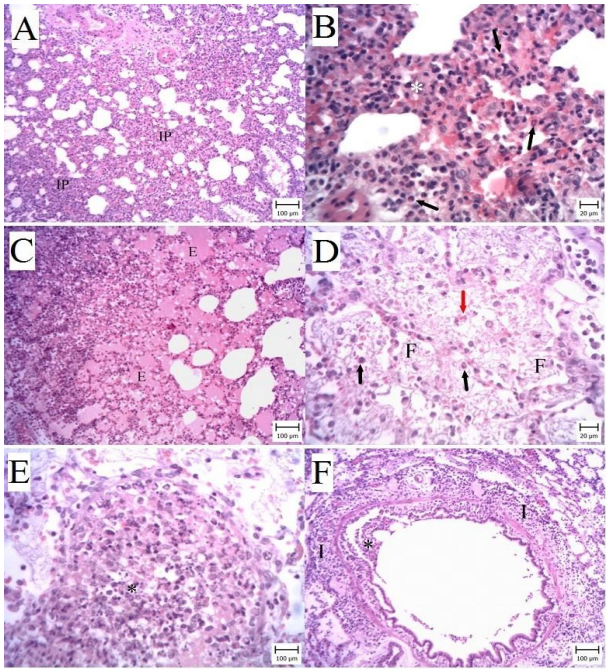

Figure 2. Rat, lung, group 2 (Cadmium exposure, sampled at day 5). (Hematoxylin and Eosin staining). **A** and **B**: Severe interstitial pneumonia. Note the thickening of the alveolar walls (asterisk) and the infiltration of inflammatory cells into the interstitial tissue and around the vessels (arrows). **C-F:** Fibrinous bronchopneumonia. **C:** Note the pink exudates inside the alveoli (E). **D:** fibrin strands inside the alveoli (F), inflammatory cells (black arrows), and erythrocytes (red arrow) are obvious. **E:** Note accumulation of necrotic cells (asterisk). **F:** accumulation of inflammatory cells around (I) and inside the bronchial (asterisk) are obvious. (Bar A, C, E, and F: 100 µm, Bar B and D: 20 µm).

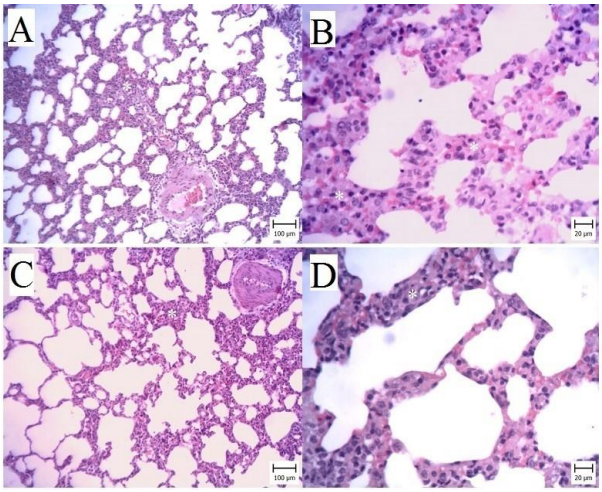

Figure 3. Lungs of rats (Hematoxylin and Eosin staining). **A** and **B:** group 5 (Low dose bromelain treatment for 24 days plus cadmium). Note the moderate interstitial pneumonia (asterisk). **C and D:** group 11 (Celecoxib treatment for 11 days). Note the mild interstitial pneumonia (asterisk) (Bar A and C: 100 µm, Bar B and D: 20 µm).

Rats in group 6, which received high dose bromelain for 19 days plus cadmium exposure, suffered from fibrinous bronchopneumonia in three cases and severe interstitial pneumonia in two other ones. High-dose bromelain treatment for 24 days in cadmium-exposed rats (groups 7) also resulted in severe interstitial pneumonia along with neutrophil infiltration in addition to fibrinous bronchopneumonia in one rat.

Rats in group 8 (high-dose bromelain treatment for 24 days) developed severe interstitial pneumonia in addition to a significant number of neutrophils in the interstitial tissue and around vessels and airways. Celecoxib treatment for 6 and 11 days plus cadmium injection (groups 9 and 10, respectively) only produced moderate interstitial pneumonia except for two rats ingroup 10 which showed severe interstitial pneumonia. Examination of the pulmonary tissue samples in control (group 1) and celecoxib-receiving (group 11) groups revealed mild to moderate interstitial pneumonia (Figure 3 C and D).

3.3. Semi-Quantitative Histopathology Results

Analysis of pulmonary pathologic indices revealed that PI pneumonia, PI infiltration, and the total index was significantly increased in cadmium-exposed groups (group 2 and 3) ($P<0.05$) (Figure 4). Treatment with low doses of bromelain for 19 and 24 days in cadmium exposed rats (groups 4 and 5) resulted in a significant reduction of all scores, compared to group 2 ($P<0.05$).However, the pathologic indices in group 4 were still significantly higher than the control group ($P<0.05$).

There was a significant decrease in PI infiltration and total index in group 6 which received high doses of bromelain for 19 days in addition to cadmium ($P<0.05$). However, the administration of the same treatment for 24 days (group 7) did not result in any significant difference in pathologic indices of group 6, compared to group 2 ($p>0.05$). Moreover, high-dose bromelain treatment alone in group 8 resulted in a significant rise in all the pathologic indices, compared to the control group ($P<0.05$). No significant change

in pathologic scores was recorded in the groups receiving celecoxib treatment with and without cadmium (group 9, 10, and 11), compared to the control group (P>0.05) (Figure 4).

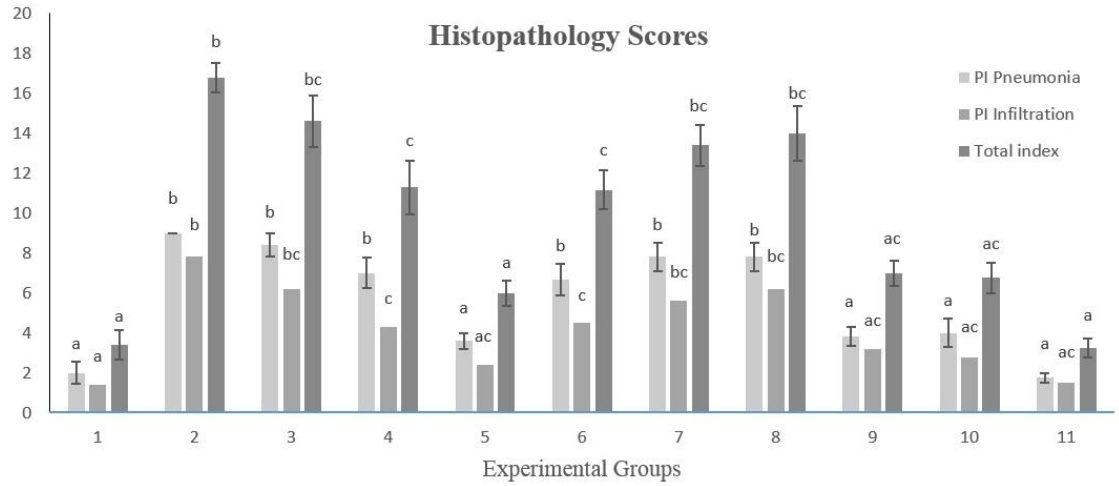

Figure 4. Histopathology semi-quantitative scores in different groups as mean±standard error. Experimental groups: 1. Control; 2 and3. $CdCl_2$, 5 and 10d, respectively; 4 & 5. Bromelain 20 + $CdCl_2$, 5 and 10d, respectively; 6 and 7. Bromelain 40 + $CdCl_2$, 5 and 10d, respectively; 8. Bromelain 40, 24 d; 9 & 10. Celecoxib+$CdCl_2$, 5 and 10d, respectively; 11. Celecoxib, 11d. Different lowercase letters above columns in each data series represent statistically significant difference between groups.

4. Discussion

Cadmium is a toxic heavy metal that can induce acute pneumotoxicity and inflammation when inhaled in high concentrations. The effects of bromelain with anti-inflammatory properties were investigated to protect against cadmium pulmonary intoxication through this study.

The cytologic analysis of BALF in the present study revealed that cadmium intratracheal instillation resulted in a significant elevation in the total nucleated cell count, epithelial cells, neutrophils, and macrophages either 5 or 10 days after the exposure. This correlates well with histopathology findings, including severe interstitial pneumonia with infiltration of neutrophils, fibrinous bronchopneumonia, necrosis and exfoliation of epithelial cells of bronchi and bronchioles, and hyperemia and hemorrhage following cadmium acute exposure. Pathologic indices of pneumonia, infiltration, and total pathologic index were also significantly increased in cadmium-exposed groups.

Cadmium-induced pulmonary damage was previously demonstrated in various experimental or clinical studies (13).In the present study, there was the coincidence of elevated total cell and neutrophil count with lung tissue involvement as well as the type of histopathologic lesions, 5 and 10 days after Cd intratracheal instillation. This was in line with findings of a previous study performed by Bergmann, Metker (14). Their study revealed that Zinc-Cadmium Sulfide (ZnCdS) exposure in rats resulted in a significantly higher white blood cell and neutrophil counts and lower macrophage count, compared to the control group at 1 and 7 days after administration.

The histopathologic assessment revealed pulmonary interstitial inflammation with thickening of alveolar septa and penetration of lymphocytes and neutrophils. Moreover, they reported subacute inflammation in the trachea and focal superficial accumulations of fibrin with captured leukocytes. Similarly, repeated cadmium inhalation was associated with a significant rise in BALF macrophages, neutrophils as well as gelatinolytic activities of some metalloproteases in rats which may progressto emphysema (15).

Moreover, Driscoll, Lindenschmidt (16) reported a significant rise in both neutrophils and lymphocytes following cadmium exposure in rats. In contrast, there

was no upsurge in alveolar macrophage counts up to 1week and once elevated, remained the same for 28 days following exposure. The principal histopathologic finding in the aforementioned study was chronic interstitial pneumonitis in which alveolar walls were thickened, mononuclear cells were accumulated, and type II cells were hyperplastic. These results are consistent with those of the present study.

Pulmonary tissue alterations in rats that were orally exposed to cadmium and mercury unaccompanied or joint were also investigated through an experimental study conducted by Koopsamy Naidoo, Bester (17). The major changes to the alveoli were collapsing, thickened septa, and infiltration of inflammatory cells while bronchioles faced morphologic changes, including smooth muscle hypertrophy as well as degenerated, detached, and aggregated epithelial cells.

Moreover, Buckley and Bassett (18) observed hyperplastic zones of epithelium, infiltration of mononuclear cells into the interstitium, and a high number of macrophages in the alveoli four days after the exposure of rats to a single cadmium oxide. Moreover, results of their experiment showed that whilst pulmonary recovery from the low-dose exposures was apparent after 15 days, lung tissues subjected to high-dose cadmium displayed a rise in non cellular thickening of the interstitial space and a persistent overall hypercellularity at the same time.

In another study, 8 weeks of cadmium exposure resulted in alveolar edema and inflammation along with airspace enlargement in mice. However, in the aforementioned study, cadmium chloride accompanied by non-steroidal anti-inflammatory drug administration decreased the inflammation which indicates the proinflammatory effects of persistent cadmium exposure even in minor amounts (19).

In addition, El-Refaiy and Eissa (20) described lung tissue changes after 90 days of $CdCl_2$ oral administration in rats which included edema, thickened alveolar walls, lymphocyte infiltration, and dilated and congested veins. They also suggested that co-administration of zinc or vitamin C can be effective in protecting lung tissues against cadmium-induced damages. Similarly, cadmium exposure resulted in alveolar collapse, granulomatous inflammatory response, thickened interstitial space, and exfoliated bronchiole epithelium in pulmonary tissue after a day, a week, and a month (21).

Saline instillation in the control group was associated with a mild to moderate interstitial pneumonia, but to a lower degree, compared to the cadmium intoxicated groups. Focal or multifocal low-intensity interstitial pneumonitis is frequent in laboratory rats which are detected incidentally and is due to minimal irritations. These forms of injuries were also noticed in this experiment but were differentiated from cadmium-generated damage by the degree of intensity and spreading. Saline intratracheal injection on its own was accompanied by the pulmonary inflammatory response which is not unusual. Bronchoalveolar lavage with sterile saline was reported to produce a temporary inflammation and can cause macrophage activation (14, 16).

Treatment with both low and high doses of bromelain for 24 days resulted in the reduction of BALF total cell count and various cell types, especially neutrophil count in cadmium-exposed rats. Although bromelain administration could not completely prevent interstitial pneumonia and fibrinous bronchopneumonia following cadmium exposure, this treatment, especially in lower doses (20 mg/kg), resulted in the significant reduction of all pulmonary pathologic scores, compared to the untreated cadmium intoxicated rats.

Bromelain treatment in this study could efficiently recover cadmium-induced bronchopulmonary inflammatory changes, especially when administered in lower doses. Bromelain anti-inflammatory properties were demonstrated earlier. Secor, Shah (22) showed significant anti-inflammatory activities in a model of allergic bronchoalveolar disease which was indicated

by reduced BALF total leukocytes, CD4+ and CD8+ T cells in addition to serum IL-4, IL-12, IL-17, and IFN-γ in the mice that received bromelain.

Although the cause is unclear, the outcomes of the administration of bromelain on BALF lymphocytes in the aforementioned study were greater when administered in lower doses (2 mg/kg, IP), compared to the higher ones (6 mg/kg). This is consistent with the findings of the present study. It was also shown that bromelain can reduce the majority of inflammatory mediators, including IL-1β, IL-6, and TNF-α in inflammation-generated excess cytokine production (23). Furthermore, bromelain was capable of modulating the production of transforming the growth factor (TGF)-β, a key regulator of inflammation in osteomyelofibrosis and rheumatoid arthritis (5).

In the present study, in the group receiving celecoxib, positive effects were observedin reducing pulmonary inflammation. Given the almost similar effects of bromelain, it is possible to attribute the protective and healing effects of bromelain against cadmium-induced pneumonia to the inhibition of the COX-2 enzyme.

Exclusive bromelain administration did not induce any significant alteration in BALF total cells and various cell types, compared to the control group. However, these rats developed severe interstitial pneumonia in addition to a significant neutrophil infiltration which was accompanied by a significant rise in all the pathologic indices, compared to the control group. Bromelain is generally believed to be safe with no adverse effects (24).

However, this plant-derived protease was reported to cause allergic reactions, including asthma, especially following occupational exposure. Results of earlier studies revealed that bromelain can induce IgE-mediated immune responses with major respiratory signs (25). Hence, the adverse pulmonary histopathologic changes in the bromelain-treated group in this study might be attributed to the probable allergic reactions following drug administration for a long period.

5. Conclusion

In total, the findings of the present study revealed that acute intratracheal exposure to $CdCl_2$ can initiate continual pulmonary histopathologic damage and inflammatory response. Furthermore, it seems that bronchopulmonary injury lasts for a minimum of 10 days following cadmium exposure indicating irreversible damage and/or reduced ability of healing. Treatment with bromelain, especially when administered in the lower dose, was effective in preventing or recovering cadmium-induced bronchoalveolar cytologic and pathologic changes. Further research is required on various aspects of bromelain administration before it can be recommended as a clinical medication in cadmium-induced pulmonary intoxication.

Authors' Contribution

Study concept and design: S. M. J., J. J. and Gh. Kh.
Acquisition of data: S. R. A. and J. J.
Analysis and interpretation of data: S. M. J. and A. R.
Drafting of the manuscript: S. M. J., A. R. and S. R. A.
Critical revision of the manuscript for important intellectual content: S. M. J. and J. J.
Statistical analysis: S. M. J.
Administrative, technical, and material support: J. J. and A. R.

Ethics

All experiments were carried out according to ethical rules for the care and use of laboratory animals and were approved by the Experimental Animals Committee of Shahid Chamran University of Ahvaz, Iran.

Conflict of Interest

The authors declare that they have no conflict of interest.

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