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. To assess the possible protective effects of bromelain against cadmium acute intratracheal exposure and its bronchopulmonary cytologic and histopathologic consequences the following treatments were performed in 11 groups of Wistar rats: Group 1 (negative control); Group 2 and 3: Cadmium Chloride (CdCl₂) 400 µg/rat intratracheally and sampled after 5 and 10 days, respectively; Group 4 and 5: bromelain 20 mg/kg orally (PO) from 14 days before until 5 and 10 days after CdCl₂ instillation, respectively; Group 6 and 7: bromelain 40 mg/kg from 14 days before until 5 and 10 days after CdCl₂ instillation, respectively; Group 8: bromelain 40 mg/kg for 24 days; Group 9 and 10: Celecoxib 25 mg/kg PO from one day before until 5 and 10 days after CdCl₂ instillation, respectively; Group 11: 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. Bromelain treatment either in low or high dose in cadmium exposed rats resulted in a significant reduction of neutrophil count. Despite that bromelain treatment could not completely prevent or recover interstitial pneumonia and fibrinous bronchopneumonia in cadmium exposed rats; low dose administration resulted in a significant decrease of semiquantitative 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 lung.

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

1. Introduction

Cadmium is a heavy metal which 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 high concentration of Cd 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

Many research findings suggest cadmium to have pro-inflammatory properties that can upregulate the inflammation mediators (4). Hence, administration of drugs/agents with antiinflammatory 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 act as an anti-inflammation and immunomodulator, as well as a wound healer and circulatory enhancer which lead to bromelain extensive administration. In addition, as a 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 nonsteroidal anti-inflammatory drug (NSAID) with analgestic, and antipyretic properties. Celecoxib belongs to a group of NSAIDs, that are considered selective COX-2 inhibitors and that are commonly known as coxibs (6). A number of 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 experiment was performed to investigate the possible protective impacts of bromelain in comparison to celecoxib, as an antiinflammatory drug, against acute intratracheal cadmium contact and its bronchopulmonary cytologic and histopathologic consequences.
2. Materials and Methods

2.1. Laboratory animals

A total of 66 Albino male Wistar rats with weigh range of 250–300 g were kept in a room with a temperature upheld at 24 ± 2 °C and a humidity of 55 ±5%, with a 12-h light/12-h dark brightness 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) and were treated as follows:

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

Group 2: 400 µg/rat Cadmium Chloride (CdCl$_2$) IT and sampled after 5 days (8).

Group 3: 400 µg/rat CdCl$_2$ IT and sampled after 10 days.

Group 4: 400 µg/rat CdCl$_2$ IT and 20 mg/kg bromelain (9) orally by gavage (PO) daily from 14 days prior to- until 5 days after cadmium instillation.

Group 5: 400 µg/rat CdCl$_2$ IT and 20 mg/kg bromelain PO daily from 14 days prior to- until 10 days after cadmium instillation.

Group 6: 400 µg/rat CdCl$_2$ IT and 40 mg/kg bromelain (10) PO daily from 14 days prior to- until 5 days after cadmium instillation.

Group 7: 400 µg/rat CdCl$_2$ IT and 40 mg/kg bromelain PO daily from 14 days prior to- until 10 days after cadmium instillation.

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

Group 9: 400 µg/rat CdCl$_2$ IT and 25 mg/kg Celecoxib (11) PO daily from one day prior to- until 5 days after cadmium instillation.
Group 10: 400 µg/rat CdCl₂ IT and 25 mg/kg Celecoxib PO daily from one day prior to- until 10 days after cadmium instillation.

Group 11: 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, so that their back was against the board and were suspended from their incisors on a wire. The tongue was softly pulled out and held to the side of the oral cavity with a 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 on dorsal position, and incised at midline from the mandible to the abdominal cavity. Afterwards, the thorax was opened and the right bronchus was ligated with nylon suture in order to prevent penetration of lavage fluid into the right lung and preserving 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 for 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 was excised. The tissues were then stored in 10% formalin fixative and the fixative was replaced after 24 h for better fixation. Afterwards, 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 semiquantitatively.

2.6. Semiquantitative histopathologic scoring method

Two independent histopathologic patterns were evaluated in this study. Firstly, cell infiltration surrounding pulmonary arteries and veins and bronchioles. Secondly, acute pulmonary inflammation that involve both the interstitial spaces and alveoli. Each pattern was semiquantitatively scored (Table 1 and 2). The severity grade was multiplied by the extent of involvement to generate a pathology index (PI) (span, 0 - 9). Eventually, the pneumonitis and periluminal infiltrate PIs were added together to calculate a total index (span, 0 - 18) (12).
2.7. **Statistical analysis**

The data was statistically analyzed using SPSS program version 16 (SPSS Inc., Chicago, IL, USA). The results were stated as mean ± standard error (SE). Nonparametric Kruskal-Wallis test was used for analysis of histopathologic and cytological data. P-value less than 0.05 represented statistical significance.

3. **Results**

3.1. **BALF Cytology results**

The comparison of total and differential cellular counts in the BALF revealed that the highest value of total cells, epithelial cells, neutrophils and eosinophils were in group 2 (Cadmium exposure, sampled at day 5) while the maximum macrophage count was observed in group 3 (Cadmium exposure, sampled at day 10) (Fig 1). Low dose bromelain treatment for 24 days in cadmium exposed rats (group 5) resulted in reduction of total count and various cell types, although this alteration was not statistically significant except in neutrophil count (p<0.05). High dose bromelain administration in cadmium received rats (group 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 BALF total cells and various cell types compared to control group, excluding lymphocyte counts in which a significant increase was observed (p<0.05). No significant change in total or differential cell counts were noted in celecoxib treatment with and without cadmium, compared to control group (p>0.05).

3.2. **Qualitative histopathology results**
In microscopic examination of the lungs of all rats of group 2 (cadmium exposure, sampled at day 5), severe interstitial pneumonia (Figure 2A) along with hemorrhage was observed. In these cases, interstitial pulmonary tissue thickness was increased. These effects were due to 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 & D). A high number of inflammatory cells, especially neutrophils, were found around the airways and vessels, inside the alveoli, bronchioles, and interstitial tissue. In addition, the accumulation of necrotic cells in the interstitial pulmonary tissue was observed in some areas (Figure 2E). Necrosis and exfoliation of epithelial cells of bronchi and bronchioles also occurred (Figures 2F). Hyperemia and hemorrhage were other microscopic features. In group 3 (cadmium exposure, sampled at day 10) and group 4 (low dose bromelain treatment for 19 days plus cadmium exposure), all rats show severe interstitial pneumonia. Fibrinous bronchopneumonia was also observed in one rat of each mentioned group. Low dose bromelain treatment for 24 days in cadmium exposed rats (groups 5) resulted in moderate interstitial pneumonia (Figure 3A and B) plus fibrinous bronchopneumonia in one case. 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 a fibrinous bronchopneumonia in one rat. Rats in group 8 (high dose bromelain treatment for 24 days) developed a 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 (group 9 and 10, respectively) only produced a moderate interstitial pneumonia except two rats of the later mentioned group in which a severe interstitial pneumonia was observed. Examination of the pulmonary tissue samples in control (group 1) and celecoxib (group 11) receiving groups revealed mild to moderate interstitial pneumonia (Figure 3 C and D).

3.3. **Semiquantitative histopathology results**

Analysis of pulmonary pathologic indices revealed that PI pneumonia, PI infiltration and total index was significantly increased in cadmium exposed groups (group 2 and 3) (p<0.05) (Fig 4). Low dose bromelain treatment for 19 and 24 days in cadmium exposed rats (groups 4 and 5) resulted in the 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). Despite a significant decrease in PI infiltration and total index in group 6 which received high dose bromelain for 19 days in addition to cadmium (p<0.05), administration of the same treatment for 24 days (group 7) did not result in any significant difference in pathologic indices 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 were recorded in celecoxib treatment with and without cadmium (group 9, 10 and 11), compared to control group (p>0.05) (Fig 4).

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 post-exposure. This correlates well with histopathology findings which included severe interstitial pneumonia with infiltration of neutrophils; fibrinous bronchopneumonia; necrosis and exfoliation of epithelial cells of bronchi and bronchioles; 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). 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, in the present study, agreed with earlier findings described by Bergman et al. (2000) (14). Their study revealed that Zinc-Cadmium Sulfide (ZnCdS) exposure in rats resulted in a significantly higher WBC and neutrophil counts and lower macrophage count compared to the control group at one day and 7 days after dosing. In histopathologic assessment, pulmonary interstitial inflammation with thickening of alveolar septa and a penetration of lymphocytes and neutrophils was observed. 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 of BALF macrophages, neutrophils as well as gelatinolytic activities of some metalloproteases in rats which may develop to an emphysema (15). Moreover, Driscoll et al. (1991) reported a significant rise in both neutrophils and lymphocytes following cadmium exposure in rats (16). In contrast, there was no upsurge in
alveolar macrophage counts up to one week and once elevated, it was persisted to 28 days following exposure. The principle histopathologic finding in the mentioned study was chronic interstitial pneumonitis in which alveolar walls were thickened, mononuclear cells were accumulated and type II cells were hyperplastic that were consistent with the outcomes of the present study. Pulmonary tissue alterations in rats orally exposed to cadmium and mercury unaccompanied or joint was also investigated through an experimental study conducted by Naidoo et al. (2019) (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 (1987) observed hyperplastic zones of epithelium, infiltration of mononuclear cells into the interstitium, and a high number of macrophages in the alveoli, four days following a single cadmium oxide exposure in rats (18). Moreover, 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 noncellular thickening of the interstitial space and a persistent overall hypercellularity at the same time. In another study, 8 week of cadmium exposure resulted in alveolar edema and inflammation along with the airspace enlargement in mice. However, 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 (2013) described lung tissue changes following 90 days of CdCl₂ oral administration in rats which included edema, thickened alveolar walls, lymphocyte infiltration, dilated and congested veins (20). They also suggested that Co-administration of zinc or vitamin C can be effective in protecting lung tissue against cadmium
produced damages. Similarly, cadmium exposure resulted in alveolar collapse, granulomatous inflammatory response, thickened interstitial space, and exfoliated bronchiol 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 than in the cadmium intoxicated groups. Focal or multifocal low intensity interstitial pneumonitis is frequent in laboratory rats which is 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 pulmonary inflammatory response and is not unusual. Bronchoalveolar lavage with sterile saline was reported to produce a temporary inflammation and can cause macrophage activation (14, 16).

Bromelain treatment for 24 days in both low and high dose resulted in 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 the lower dose (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 Cd induced bronchopulmonary inflammatory changes, especially when administered in the lower dose. Bromelain anti-inflammatory properties were demonstrated earlier. Bromelain showed a significant anti-inflammatory activity 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 bromelain-received mice (22). Although the cause is unclear, the outcomes of bromelain administration on BALF lymphocytes in the last mentioned study, were greater when administered in the lower dose (2 mg/kg, IP), compared to the higher one (6 mg/kg) which was 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 to modulate the production of transforming growth factor (TGF)-β, a key regulator of inflammation in osteomyelofibrosis and rheumatoid arthritis (5).

In the group receiving celecoxib in the present study, positive effects in reducing pulmonary inflammation were observed, and given the almost similar effects of bromelain, it is possible to attribute the protective and healing effects of bromelain against cadmium-induced pneumonia to 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 control group. However, these rats developed a 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. 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.

### 4.1. Conclusion

In total, findings in the present study revealed that acute intratracheal exposure to CdCl2 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. Bromelain treatment, 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.

### 5. Authors' Contribution

Study concept and design: S. M. J., J. J., G. K.

Acquisition of data: S. R. A., J. J.

Analysis and interpretation of data: S. M. J., A. R.

Drafting of the manuscript: S. M. J., A. R., S. R. A.

Critical revision of the manuscript for important intellectual content: S. M. J., J. J.

Statistical analysis: S. M. J.

Administrative, technical, and material support: J. J., A. R.
6. **Ethics**

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

7. **Conflict of Interest**

The authors report no conflict of interest

8. **Grant support**

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9. **Acknowledgment**

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10. **References**

Figure 1. Cytologic results of BALF in different groups as mean ± SE. Experimental groups: 1. Control; 2 & 3. CdCl₂, 5 and 10d, respectively; 4 & 5. Bromelain 20 + CdCl₂, 5 and 10d, respectively; 6 & 7. Bromelain 40 + CdCl₂, 5 and 10d, respectively; 8. Bromelain 40, 24 d; 9 & 10. Celecoxib + CdCl₂, 5 and 10d, respectively; 11. Celecoxib, 11d. Different lowercase letters above columns in each data series represent statistically significant difference between groups.
Figure 2. Rat, lung, group 2 (Cadmium exposure, sampled at day 5). (Hematoxylin and Eosin staining). A & B: Severe interstitial pneumonia (IP). 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 bronchiol (asterisk) are obvious. (Bar A, C, E & F: 100 µm, Bar B & D: 20 µm).
Figure 3. Rat. Lung. (Hematoxylin and Eosin staining). A & B: group 5 (Low dose bromelain treatment for 24 days plus cadmium). Note the moderate interstitial pneumonia (asterisk). C & D: group 11 (Celecoxib treatment for 11 days). Note the mild interstitial pneumonia (asterisk) (Bar A & C: 100 µm, Bar B & D: 20 µm).
Figure 4. Histopathology semiquantitative scores in different groups as mean ± SE. Experimental groups: 1. Control; 2 & 3. CdCl₂, 5 and 10d, respectively; 4 & 5. Bromelain 20 + CdCl₂, 5 and 10d, respectively; 6 & 7. Bromelain 40 + CdCl₂, 5 and 10d, respectively; 8. Bromelain 40, 24 d; 9 & 10. Celecoxib + CdCl₂, 5 and 10d, respectively; 11. Celecoxib, 11d. Different lowercase letters above columns in each data series represent statistically significant difference between groups.