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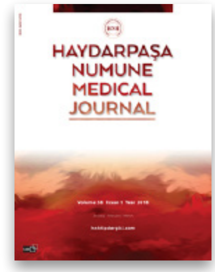

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ORIGINAL ARTICLE

Antioxidant Effects of Bisphosphonates in Smoking-Induced Lung Injury in a Rat Model

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Abstract

Introduction: The role of smoking in the development of lung injury is well established. Many studies have reported that the oxidative stress is increased in smokers. Previous studies have investigated the oxidant and antioxidant effects of zoledronic acid in tissues such as hepatic and oral epithelial cells, but not in lung tissue and bronchial lavage.

Methods: The rats were divided into two groups: the first group was exposed to cigarette smoke (CS), and the second group was given subcutaneous zoledronic acid along with cigarette smoke exposure (ZCS). The lung tissue analysis of the groups included interstitial fibrosis, emphysema, and lymphocyte response at the interstitial space. In serum and bronchoalveolar lavage fluid, superoxide dismutase activity and levels of the antioxidant, lipid hydroperoxide, glutathione, H_2O_2, and transforming growth factor beta1 were measured.

Results: The accumulation rate of fibroblasts at the interstitial space was significantly higher in the CS group. The bronchoalveolar lavage levels of H_2O_2 used for the assessment of oxidative stress were significantly lower in the ZCS group.

Discussion and Conclusion: The present study showed that zoledronic acid was effective in reducing the smoke-induced oxidative stress in the lung, especially by strongly lowering the bronchoalveolar lavage levels of H_2O_2, and it reduced the fibroblast proliferation in the interstitial space.

Keywords: Antioxidant; bisphosphonate; oxidative stress.

Chronic smoke exposure causes airway and lung parenchymal inflammation characterized by increased numbers of macrophages, lymphocytes, neutrophils, and/or eosinophils [1]. There are many studies reporting that the oxidative stress is increased in smokers. Cigarette smoke contains oxygen-derived radicals. While many studies have shown increased production of free radicals in the phagocytic cells of smokers, a group of studies has shown a reduction in certain antioxidants in COPD. Compared with healthy controls, COPD patients had elevated levels of lipid peroxidation products during an acute attack. Therefore, an oxidant–antioxidant imbalance may occur, leading to excessive oxidant stress, playing a significant role in the pathogenesis of COPD in smokers [2]. Cigarette smoke contains higher concentrations of reactive oxygen species (ROS) such as hydroxyl radical (OH-) and superoxide anion

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(O_2.-). The semiquinone radicals present in the particulate phase of the cigarette smoke are responsible for the production of ROS and reactive nitrogen species in the lungs. These free radicals destroy biologically important molecules and reduce members of the antioxidant system. While metals in the cigarette smoke such as chromium, nickel, copper, and iron cause increase production of ROS, other metals such as copper, cadmium, and mercury deplete thiol-containing antioxidants and suppress antioxidant enzyme activity. ROS in cigarette smoke reacts with antioxidants found in the cell membranes and airways, leading to cell damage in the lungs due to oxidative stress in case of the dominance of the oxidants. Cigarette smoke has a triggering role in various cellular events that occur via several ways such as apoptosis, inflammation and gene expression [3]. It also contributes to the development of interstitial lung diseases [4].

Bisphosphonates inhibit bone resorption and are indicated for the treatment of osteoporosis including disuse osteoporosis all over the world. Zoledronic acid, which is a bisphosphonic acid, is a heterocyclic nitrogen-containing bisphosphonate that has an imidazole-ring side chain. Being a third-generation bisphosphonate, zoledronic acid is 10.000 times more active than the first-generation etidronate [5]. Zoledronic acid inhibits osteoclasts as well as inhibiting bone resorption, as previously described in general for bisphosphonates. The reported potential mechanisms of action of zoledronic acid include inhibition of osteoclast maturation, inhibition of osteoclast recruitment to the site of bone resorption [6], suppression of mature osteoclast function [7], reduction of IL–6 cytokine production [8], direct antitumor activity (cytostatic and cytolytic) [9], inhibition of tumor-cell dissemination, invasion, and adhesion to the bone matrix [10], and anti-angiogenic activity [11]; however studies on the antioxidant and anti-nflammatory effects of zoledronic acid are limited and have controversial results.

Previous studies have investigated the oxidant and antioxidant effects of zoledronic acid in tissues such as hepatic and oral epithelial cells, but not in lung tissue and bronchial lavage. The present study aimed to evaluate the antioxidant and anti-inflammatory effects of zoledronic acid in lung tissue, blood, and bronchoalveolar lavage for development of any lung injury caused by the harmful effect of the cigarette smoke-induced oxidative stress in a rat model.

Materials and Methods

The study was carried out in the experimental animal laboratory at experimental medical research and application center of the medical faculty. Ethical approval was provided by the Local Ethics Committee, Our study was planned in accordance with the guidelines of animal ethics and welfare [12]. Twenty male Wistar strain albino rats weighing between 510 and 580 g (mean 550 g) were used as experimental animals. The animals were housed in cages with five rats per cage and fed a standard laboratory diet and water. They were randomly divided into two groups; 10 in cigarette smoke (CS) exposure group and 10 in zoledronic acid plus cigarette smoke exposure (ZCS) group. All subjects were exposed to inhalation of cigarette smoke for 4 weeks. Rats were exposed to the cigarette smoke for 30 minutes twice a day in a plastic container (40 cm x 26 cm x 16 cm) using a bellows system. The nicotine content of the cigarette used was 1 mg. The zoledronic acid group initially received 15 µg/kg subcutaneous zoledronic acid based on the average dose used for adult humans. The control group received subcutaneous injection of normal saline. At the end of 4 weeks, all subjects were administered intraperitoneal ketamine 60 mg/kg and xylazine HCl 30 mg/kg and were sacrificed. A 5 cc blood was drawn using the intracardiac route, and a bronchial lavage was performed with 5 cc saline using the tracheal route. The blood samples were centrifuged at a rate of 3,000 g at +4°C temperature for 10 min to separate serums. The bronchoalveolar lavage fluid was centrifuged at a rate of 420 g at +4°C for 10 min to separate supernatants. Serum and bronchoalveolar lavage fluid were stored at –80°C until used for testing.

In serum and bronchoalveolar lavage fluid, superoxide dismutase (SOD) activity and levels of the antioxidant, lipid hydroperoxide (LPO), glutathione (GSH), and hydrogen peroxide were measured using a spectrophotometric kit (Cayman, Michigan, USA). Spectrophotometric measurements were performed using the Bio-Rad microplate absorbance reader xMark (Bio-rad Laboratories, California, USA) based on the absorbance-concentration and calibration graphics. In serum and bronchoalveolar lavage fluid, the transforming growth factor (TGF) beta1 level was measured using an ELISA kit (Ebioscience, Vienna, Austria). The ELISA kit measurement was performed using an ELx50 microplate washer and ELx800 absorbance reader based on the absorbance-concentration and calibration graphics. The lungs were fixed in 10% formol saline for tissue analysis. After they had been embedded in paraffin, 5-µm sections were taken with a microtome and stained with hematoxylin and eosin and evaluated for lung injury. The evaluation included interstitial fibrosis, emphysema, lymphocyte response around bronchi and bronchioles and the type and amount of cells recruited at the interstitial space. A semi-quantitative grading system described by Ashcroft was used for assessment of interstitial fibrosis [13]. In this grading system, the score ranges from 0 (normal) to 8 (total fibrosis). Other parameters were evaluated to be using another semi-quantitative scoring system [14].

Statistical Analysis

We used SPSS statistics 17.0 software, chi-square test to compare categorical variables, and Mann–Whitney U-test to compare continuous variables between groups. The significance level was set at $p<0.05$.

Results

The severity of the interstitial fibrosis which was similar in both groups was Grade 1 according to the Aschoft's criteria. Emphysema was more in the ZCS group, but all were focal areas and were mildly rated. No statistically significant difference was found between the two groups (Table 1). Similarly, there was no statistically significant difference between the two groups in the assessment of the lymphocytic response around bronchi and bronchioles (Table 1).

Table 1. Histopathological analysis of the lung tissue

	CS n	ZCS n	p
Fibrosis*	2	2	1.00
Emphysema**	5	8	0.16
Lymphocytic response**			
Mild	3	2	0.86
Moderate	5	6	
Pronounced	2	2	
Cell accumulation at interstitial space**			
Focal mild	2	6	
Focal marked***	8	2	0.02
Diffuse mild	0	2	

*=Ashcroft scoring system; **=Semiquantitative scoring system; ***=The group that created the differance; CS: Cigarette smoke exposure; ZCS: Zoledronic acid plus cigarette smoke exposure.

Table 2. Types of cells in the interstitial space in histopathological evaluation

	CS n	ZCS n	p
Macrophages	60.5±12.12	62±15.67	0.79
Fibroblast	21.5±7.83	9.5±4.37	0.02
Lymphoplasmacytic cell	15±5.270	24.5±13.42	0.12
Neutrophil polymorphs	3±4.21	4±6.58	0.97

CS: Cigarette smoke exposure; ZCS: Zoledronic acid plus cigarette smoke exposure.

An analysis of the cell accumulation in the interstitial space showed higher focal mild accumulation in the ZCS group, but the difference was not statistically significant. However, marked cell accumulation in focal areas was higher in CS group with a statistically significant difference (p=0.02) (Table 1, Fig. 1).

An analysis of the number of cells in the interstitial space showed that the number of fibroblasts was significantly higher in the CS group (Table 2, Fig. 2).

The assessment of oxidative stress in blood and bronchoalveolar lavage fluid included superoxide dismutases (SOD), reduced glutathione (GSH), lipid hydroperoxide, hydrogen peroxide, and antioxidant kit, and the assessment of anti-inflammatory effect included concentrations of

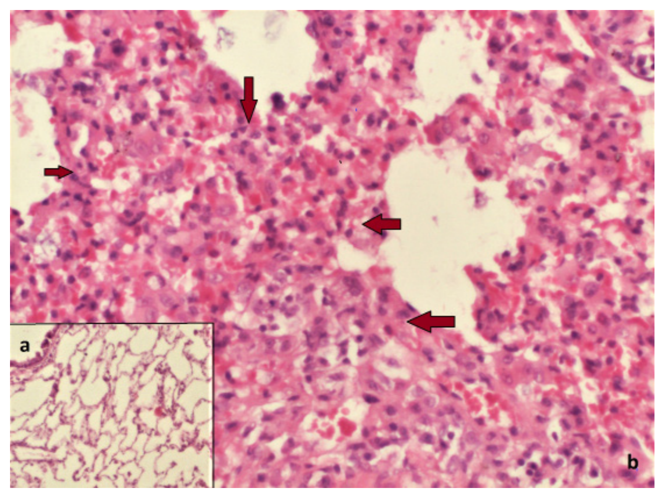

Figure 1. x100, H&E, normal lung **(a)**; Increased cell accumulation in the interstitial space in CS group **(b)**.

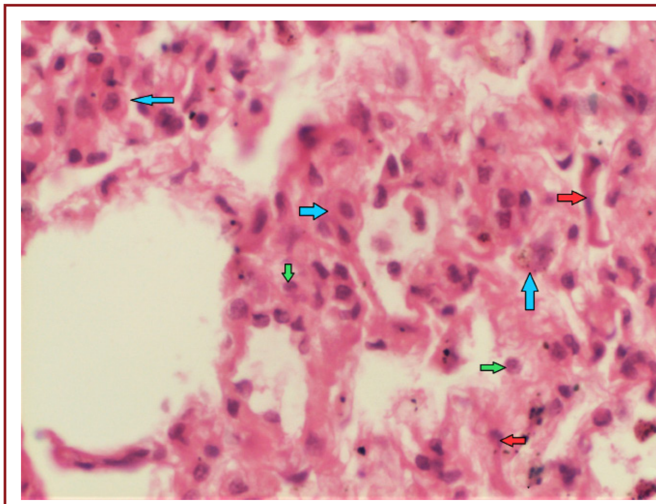

Figure 2. x400, H&E, Types of cells in the interstitial space; fibroblasts (red arrow), macrophages (blue arrow) and rare lymphoplasmacytic cells (green arrow), in CS the group.

transforming growth factor beta (TGF-β). The levels of antioxidant capacity in blood were higher and hydrogen peroxide (H_2O_2) levels were lower in the ZCS group than in the CS group. However, no statistically significant difference was found (Table 3).

In bronchoalveolar lavage, the H_2O_2 value was 0.0914±0.0753 µM in the CS group, while it was lower in the ZCS group (0.0045±0.0037 µM) with a statistically significant difference (Table 4).

Discussion

To the best of our knowledge, no study has been published on the oxidant and antioxidant efficacy of zoledronic acid in the lungs until today. In the meantime, the results from studies investigating such efficacy of zoledronic acid in other organs and tissues are controversial.

A study by Karabulut et al. [15] of the hepatic tissue of rabbits found significantly lower GSH levels than the control group. In the same study, histopathological analysis of the hepatic tissue of rabbits showed no significant difference with the control group. In the present study, there was no significant difference between the CS and ZCS groups in SOD vs GSH levels in blood and bronchoalveolar lavage. However, although histopathological analysis of the lung tissue showed no significant difference in emphysema, fibrosis and the lymphocytic response around bronchi and bronchioles, the cell accumulation in interstitial space and rate of fibroblast cells were significantly higher in the CS group.

Table 3. Assessment of oxidant, anti-oxidant and anti-inflammatory parameters in blood

	CS	ZCS	p
SOD, U/ml	0.889±0.25	0.818±0.34	0.70
TGF Beta1, pg/ml	246.100±84.54	243.700±65.16	0.73
Antioxidant, mM trolox	2.829±2.14	4.266±2.08	0.19
LPO, nmol	0.798±0.69	0.831±0.92	0.68
GSH, µM	49.460±29.33	49.330±27.35	1.00
H_2O_2, µM	0.617±0.45	0.450±0.20	0.73

CS: Cigarette smoke exposure; ZCS: Zoledronic acid plus cigarette smoke exposure; SOD: Superoxide dismutase; TGF: Transforming growth factor; LPO: Lipid hydroperoxide; GSH: Glutathione; H_2O_2: Hydrogen peroxide.

Table 4. Assessment of oxidant, anti-oxidant and anti-inflammatory parameters in bronchoalveolar lavage

	CS	ZCS	p
SOD, U/ml	0.0026±0.0017	0.0028±0.0036	0.52
TGF Beta1, pg/ml	39.1000±11.3377	43.80±10.0421	0.28
Antioxidant, mM trolox	0.1080±0.0682	0.1240±0.1125	0.97
LPO, nmol	0.6110±0.8529	0.2670±0.1750	0.28
GSH, µM	5.2300±0.9165	4.6000±1.3114	0.28
H_2O_2, µM	0.0914±0.0753	0.0045±0.0037	0.001

CS: Cigarette smoke exposure; ZCS: Zoledronic acid plus cigarette smoke exposure; SOD: Superoxide dismutase; TGF: Transforming growth factor; LPO: Lipid hydroperoxide; GSH: Glutathione; H_2O_2: Hydrogen peroxide.

Small numbers of interstitial cells also reside in the connective tissue spaces such as mast cells, fibroblasts, myofibroblasts, macrophages, and plasma cells. Any injury in the lung tissue by a harmful factor induces inflammatory and repair processes. If it is a long-term and intense effect, the process of repair is prolonged with the effect of proinflammatory and profibrotic cytokine releases by inflammatory cells, proliferating epithelial cells and matrix components. These uncontrolled proliferation results in collagen deposition, the proliferation of fibroblasts and thickening of the pulmonary capillaries. As it becomes chronic, interstitial and intra-alveolar fibrosis and alveolar collapse develop [16]. In the present study, the fibroblast proliferation in interstitial space was 21.5%±7.83% in the CS group vs. 9.5±4.37% in the ZCS group (p=0.02).

A study by Tuğ et al. [17] reported that biomolecular and inflammatory events exacerbated by oxidative stress which become more intense during COPD attacks have significant and negative effects on the course and prognosis of the disease; despite increasing excessive oxidant load and negative impacts during attacks, essential antioxidant systems (GSH and GSH-Px) which can play a role as an eliminator of the oxidant damage, maintain their level, even they show no increase; and the need for antioxidant support is increased due to the increased oxidative load, which becomes more pronounced during COPD exacerbations, and thus a novel therapy is required for treatment of COPD. In the present study, remarkably lower rates of H_2O_2, an important indicator of oxidative stress, in the ZCS group in blood and particularly in bronchoalveolar lavage despite lack of significant difference in antioxidant parameters between the two groups provide support to these authors' conclusion, and indicate that zoledronic acid has a strong antioxidant efficacy in the lungs.

In a study of TGF-β by Li et al. [18] in the regulation of immune response, TGF-β was reported to be one of the most potent immunosuppressive molecules, and TGF-β suppresses the immune and inflammatory response by suppressing the activity of effector T (Th1 and Th2) and cytostatic T cells of the immune system, and activating regulatory T cells (Tregs). Administration of bisphosphonates (BF) results in increased secretion of some cytokines. A study by Naidu et al. [19] examined the effect of zoledronic acid on TGF-β and found increased secretion of TGF-β from osteoblasts. In the present study, although not statistically significant, TGF-β was increased in the ZCS group compared to the CS group in bronchoalveolar lavage (39.1000±11.3377 pg/ml and 43.80±10.0421 pg/ml, respectively). It suggests that zoledronic acid may indirectly contribute to the anti-inflammatory process through TGF-β.

In addition, Pons et al. [20] found that macrophage proliferation due to development of inflammation in COPD patients was reduced compared to smoking patients without COPD (macrophages; 94.1% in smoking patients with normal respiratory functions, and 90.2% in COPD patients). In the present study, macrophages were 60.5% ± 12.12% in the CS group vs. 62±15.67 in the ZCS group. It suggests that zoledronic acid may have a protective effect against COPD although statistically non-significant.

Conclusion

In the present study, the fibroblast proliferation in interstitial space in the CS group higher than ZCS group. This result suggests that zoledronic acid may have a protective effect in the development of fibrosis. In our study, an H_2O_2 level, one of the major indicators of oxidative stress, was remarkably higher in the CS group than in the ZCS group in the bronchoalveolar lavage. The results showed that zoledronic acid was effective in reducing the smoke-induced oxidative stress, especially by strongly lowering the bronchoalveolar lavage levels of H_2O_2, and it reduced fibroblast proliferation in the interstitial space, however, longer and more comprehensive studies are required to show other possible histopathological effects.

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Conflict of Interest: The authors declare that there is no conflict of interest in preparing this article.

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