

# Human T-Cell Leukemia Virus Type 1 Changes Leukocyte Number and Oxidative Stress in the Lung and Blood of Female BALB/c Mice

## Abstract:

**Background:** Human T-cell leukemia virus type 1 (HTLV-1) infection is likely to induce nonneoplastic inflammatory pulmonary diseases. Therefore, an experimental study was conducted to evaluate the leukocytes' number alteration and oxidative stress in the lung and blood of HTLV-1-infected BALB/c mice, which could be of benefit for the recognition of HTLV-1 mechanism in the induction of pulmonary disorders. **Materials and Methods:** Twenty female BALB/c mice were divided into two groups of control and HTLV-1-infected animals. The HTLV-1-infected group was inoculated with  $10^6$  MT-2 HTLV-1-infected cells. Two months later, the infection was confirmed using real-time polymerase chain reaction, and then lung pathological changes, total and differential inflammatory cell counts in the blood and bronchoalveolar lavage fluid (BALF), along with oxidative stress biomarker levels in the BALF and lung tissue were evaluated. **Results:** In the HTLV-1-infected group, the peribronchitis score ( $P < 0.01$ ), the number of total leukocytes, neutrophils, lymphocytes, and monocytes ( $P < 0.05$ ) in the blood and BALF were increased. The number of eosinophils in the blood of the HTLV-1-infected group was higher than in the control group ( $P < 0.01$ ), whereas the number of basophils of BALF was increased in the HTLV-1-infected group ( $P < 0.001$ ). The lung and BALF oxidative stress results showed that the MDA level was increased, while the total thiol level and superoxide dismutase activity were decreased in the HTLV-1-infected group ( $P < 0.01$ ). **Conclusion:** The HTLV-1 infection seems to induce pulmonary inflammatory reactions by recruiting leukocytes as well as inducing oxidative stress in the lung tissue.

**Keywords:** BALB/c, Mice, bronchoalveolar, Human T Cell Leukemia Virus I, oxidative stress

## Introduction

Human T-cell leukemia virus type 1 (HTLV-1) belongs to the family of vertebrate retroviruses (*Retroviridae*) that is endemic in Brazil, Japan, the Caribbean, and Iran; 2%–7% of the people of Khorasan Razavi Province, in the northeast of Iran, are infected with this virus.<sup>[1]</sup> HTLV-1 is linked to two life-threatening illnesses, HTLV-1-associated myelopathy (HAM)/tropical spastic paraparesis (TSP) and HTLV-1-associated malignancy (adult T-cell leukemia/lymphoma [ATLL]).<sup>[2]</sup> Furthermore, some ATLL patients and HTLV-1 carriers regularly exhibit pulmonary complications characterized by one of the following forms: lymphocytic interstitial pneumonitis (LIP), alveolitis,<sup>[3]</sup> HTLV-1-associated bronchioloalveolar disorder (HABA), bronchopneumopathy,<sup>[4,5]</sup> diffuse panbronchiolitis-like disease (DPB),

and bronchiectasis.<sup>[3]</sup> HTLV-1-associated bronchiectasis is particularly common in the Australian population.<sup>[6]</sup> The DPB is a severe obstructive respiratory disorder recognized by the appearance of injuries in the respiratory bronchiole.<sup>[7]</sup> Furthermore, bronchiolitis as a chronic complication is characterized by lymphocyte accumulation in the lung bronchi.<sup>[6]</sup> Yamazato confirmed the tendency to bronchopulmonary disorders in some of the HTLV-1 carriers.<sup>[8]</sup> The oncoprotein of p40 Tax as a product of the HTLV-1 pX region has a vital role in the generation of pulmonary disturbances, for example, the development of inflammatory pulmonary diseases in p40 tax-HTLV-1 transgenic mice.<sup>[9]</sup>

Interestingly, some previous studies have been reported that HTLV-1 alters the oxidant-antioxidant balance by increasing the production of reactive oxygen species (ROS) or by destroying antioxidants.<sup>[10,11]</sup> A reduction in the serum

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general antioxidant potential in HTLV-1-infected individuals has been reported.<sup>[10]</sup> Furthermore, our previous study has shown that oxidative stress increases in the hippocampal and cortical tissues of HTLV-1-infected mice.<sup>[11]</sup> Oxidative stress plays a fateful role in the progression of different lung disorders, including LIP.<sup>[12]</sup> ROS, including hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and superoxide anion, are able to damage the lung tissue.<sup>[13]</sup>

In addition to oxidative stress, phenomena including immunologic mechanisms and inflammatory cell migration play critical roles in the pathogenesis of HABA<sup>[4]</sup> and LIP.<sup>[14]</sup> The complicated phenomenon of lymphocytic infiltration toward peribronchial tissues and lung perivascular spaces is considerable in p40 tax-HTLV-1 transgenic mice.<sup>[9]</sup> Expression of Tax is connected with inflammatory cell chemotaxis induced by raised chemokine secretion.<sup>[8,15]</sup> The collected bronchoalveolar lavage fluid (BALF) of individuals including HTLV-1-infected carriers with chronic respiratory inflammatory diseases, ATLL, and HAM/TSP patients contains raised levels of CD3+/CD25+ lymphocytes.<sup>[16]</sup>

Nevertheless, in previous studies, histopathological and biochemical changes caused by HTLV-1 infection in the respiratory system were not recognized clearly. Therefore, this study evaluated the total white blood cell (WBC) count and differential WBC count in the BALF and blood of HTLV-1-infected female BALB/c mice. Furthermore, in this study, oxidative stress biomarkers, including malondialdehyde (MDA) and total thiol level, superoxide dismutase (SOD), and catalase (CAT) activities, as well as histopathological changes in the lungs, were assessed.

## Materials and Methods

### Human T-cell leukemia virus type 1-infected T-lymphocyte cell line

HTLV-1-infected human T-lymphocyte cell line named MT-2 cells<sup>[17]</sup> was used in this study. MT-2 cells were originated from the co-culturing of human normal leukocytes with ATLL T-cells. Incubation of MT-2 cells was accomplished at 37°C and a 5% CO<sub>2</sub> humid incubator in the RPMI medium 1640 plus 0.1% penicillin-streptomycin combination, 1% glutamine, and 10% fetal calf serum.

### Laboratory animals

Female BALB/c mice (20–30 g, 4–6 weeks of age) were reared under normal warehouse conditions at 22°C–24°C, 12 h artificial light-dark cycle, and with free access to standard mice chow and water. All the experiments were performed based on the guiding principles for animal care and use approved by the Ethics in Research Committee, Mashhad University of Medical Sciences.

The mice were randomly assigned to two groups of control, injected phosphate-buffered saline (PBS) solution intraperitoneally, and the HTLV-1-infected group, injected

with MT-2 cells (10<sup>6</sup> cells counted) dissolved in the PBS solution.

### Assessment of human T-cell leukemia virus type 1 infection

To assess the HTLV-1 infection, HTLV-1 proviral DNA load was evaluated briefly. Peripheral blood mononuclear cells (PBMCs) were separated from whole blood samples treated with ethylenediaminetetraacetic acid (EDTA). Real-time polymerase chain reaction (PCR) for the mice was performed using a real-time PCR-based absolute quantification kit (Novin Gene, Iran) by a Rotor-Gene Q 6000 machine (Corbett Research, Australia). Then, reaction threshold cycles (CTs) were taken as confirmation of mice infection.

### Primers and probes for real-time polymerase chain reaction

To quantify the HTLV-1 proviral DNA load, forward primer (SK110 (5'-CCCTACAATCCAACCAGCTCAG-3', HTLV-1 nucleotide 4758-4779), reverse primer (SK111 (5'-GTGGTGAAGCTGCCATCGGGTTTT-3', HTLV-1 nucleotide 4943-4920), and an HTLV-1 TaqMan probe (5'-CTT TACTGACAAACCCGACCTACCC ATGGA-3) were utilized. The probe associated with the FAM reporter dye in 5' side and BHQ-1 quencher dye in 3' side was placed between positions 4829 and 4858 of the HTLV-1 genome<sup>[11]</sup> (GenBank primer accession no. J02029). The primers of Alb-AS (5'-GACCATACGTGAAGACCTAA-3'), Alb-S (5'-CCTTGTCAGTAGATGCAAAG-3') and TaqMan probe (5'-FAM-CACATCACAACCACAACCTTCTCAG-BHQ1-3') are associated with the housekeeping gene of albumin and were used in this study.

### Bronchoalveolar lavage fluid preparing and count of total white blood cell and differential white blood cell in bronchoalveolar lavage fluid and blood

The trachea and lungs were dissected and washed with distilled water. The right lung was clamped; the trachea was cannulated, and the left lung was lavaged five times, with 1 ml of saline at room temperature. After collecting BALF, the right lung was dissected and placed in a 10% formalin solution (37% formaldehyde) for pathological evaluation. The WBC content of BALF and cardiac blood samples was observable and countable by the microscope, hemocytometer technique, and Turk's staining technique for WBC total and Wright-Giemsa staining technique for WBC differentiation. Biochemical assays were performed on the left lung and the isolated supernatant of BALF.

### Biochemical assays

#### Assessment of malondialdehyde

The MDA concentration in the BALF and left lung tissue of animals in both groups was determined in accordance

with the method explained by Mihara and Uchiyama.<sup>[18]</sup> The MDA level is a criterion of tissue lipid peroxidation. In this method, the MDA in BALF or left lung tissue homogenate at 100°C was reacted with a thiobarbituric acid, which is a mixture of hydrochloric acid and trichloroacetic acid. Within 35–45 min, a reddish coloring complex (absorbance peak wavelength: 535 nm), as the MDA concentration index, was produced that was determined by the calorimetric method.

*Assessment of content of total thiol*

In accordance with Sedlak and Lindsay’s method,<sup>[19]</sup> thiol groups in some intra- or extracellular antioxidants are able to react with 2,2’-dinitro-5,5’-dithiodibenzoic acid (DTNB) and produce a yellowish complex that is detectable and measurable. Based on this method, the first absorbance of the mixture of BALF or lung tissue homogenate (0.5 ml) plus the EDTA solution (1 ml) was determined at a 412 nm absorbance peak wavelength for the yellowish complex. Reading of the absorbance of the mixture was repeated after the reagent of DTNB (20 µl) was added to the former mixture and the incubation time (15 min) passed at room temperature (37°C).

*Assessment of superoxide dismutase*

In this research, the SOD assessment method introduced by Madesh and Balasubramanian<sup>[20]</sup> was employed. Accordingly, pyrogallol and tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide were added to the BALF or lung tissue homogenate. Tissue SOD destroys superoxide radicals that are the result of pyrogallol oxidation. In this method, a reduction in the amount of tetrazolium dye characterized at the absorbance peak wavelength of 535 nm is an index of the SOD amount.

*Assessment of catalase*

The CAT assessment was performed according to Aebi’s method<sup>[21]</sup> in accordance with the downtrend of absorbance of the mixture BALF or lung tissue homogenate and H<sub>2</sub>O<sub>2</sub> because of the decomposition of H<sub>2</sub>O<sub>2</sub> by tissue CAT at 240 nm. The amount of CAT able to degrade H<sub>2</sub>O<sub>2</sub> (1 µmol) for 1 min is introduced as a unit (U) of the enzyme.

**Lung histopathological evaluation**

For histopathological examination, the formalin solution-fixed right lung tissues were embedded in paraffin and the paraffin casting was sectioned (4 µm) using an Autotechnicon device. The hematoxylin and eosin method was used for staining the tissues. The pathological changes in the right lung of control and infected groups, including peribronchitis, bronchitis, lymphoid follicles, and necrosis, were assessed. The pathological changes in most parts of

the right lung were recorded according to the following: 0: normal or without change, 1: patchy, 2: local, 3: scattered, and 4: severe changes.<sup>[22]</sup> All the chemicals used for oxidative stress and **histopathological** analyses were purchased from Merck Company, Germany.

**Statistical analysis**

The statistical analyses were performed in SPSS version 11. Mean ± standard error of the mean was used for result description. The parametric Student’s two-sample *t*-test was applied for comparing normally distributed data and the Mann–Whitney U-test for comparing the lognormal distribution data of two independent samples. *P* < 0.05 was accepted as statistically significant.

**Results**

**Human T-cell leukemia virus type 1 infection confirmation**

The infection of the mice with HTLV-1 was confirmed based on the CTs [Table 1] of amplified PBMC DNA obtained by real-time PCR assays.

**Lung histopathology**

The score of peribronchitis in the HTLV-1-infected group was significantly higher than in the control group [*P* < 0.01, Figure 1 and Table 2]. There was a nonsignificant increase in bronchitis in the HTLV-1-infected group as compared to the control group. There was no difference in lymphoid follicles between the two groups, and no necrosis was observed in the tissues.

**The effect of human T-cell leukemia virus type 1 infection on total and differential white blood cell in blood**

Total WBC count [*P* < 0.001, Figure 2a], the number of monocytes [*P* < 0.001, Figure 2b], lymphocytes [*P* < 0.05, Figure 2c], eosinophils [*P* < 0.01, Figure 2e], and neutrophils [*P* < 0.01, Figure 2f] in the blood of the HTLV-1-infected group were significantly higher than in the control group. A negligible increase in the blood basophil number of the HTLV-1-infected group was observed [Figure 2d].

**The effect of human T-cell leukemia virus type 1 infection on total and differential white blood cell in bronchoalveolar lavage fluid**

Total WBC count [*P* < 0.001, Figure 3a], the number of monocytes [*P* < 0.05, Figure 3b], lymphocytes [*P* < 0.05, Figure 3c], basophils [*P* < 0.001, Figure 3d], and neutrophils [*P* < 0.001, Figure 3f] in the BALF of the HTLV-1-infected group were significantly higher than in the control group. A negligible increase in the eosinophil

**Table 1: CTs for DNA HTLV-1 in total DNA extraction of PBMCs of HTLV-1 infected group**

Number infected mice	1	2	3	4	5	6	7	8	9	10
CTs	35.36	35.15	37.40	35.01	35.42	37.83	34.66	34.64	33.98	30.77

number in the BALF of the HTLV-1-infected group was also observed [Figure 3e].

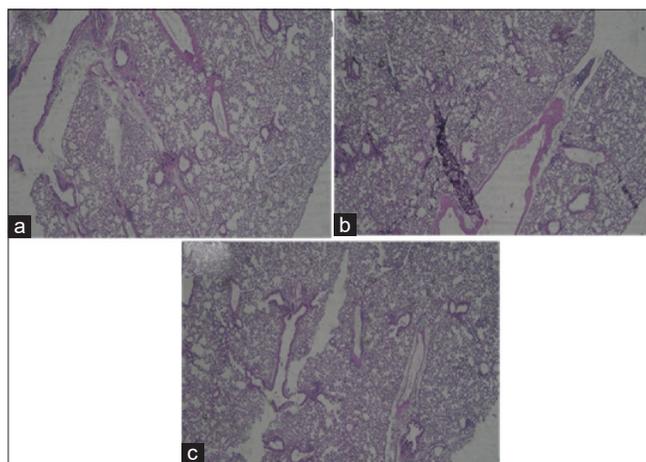


Figure 1: Photographs of a lung specimen in the control (a) and human T-cell leukemia virus type 1-infected groups (b and c), increased inflammatory infiltrate intra interstitial component of lung (b and c) (magnification for each group;  $\times 40$ ,  $\times 10$ )

### The effect of human T-cell leukemia virus type 1 infection on malondialdehyde and total thiol content, catalase, and superoxide dismutase activities in lung tissue and bronchoalveolar lavage fluid

In the lung tissue and BALF of the HTLV-1-infected group [Table 3], MDA concentration was higher than in the control group ( $P < 0.01$ ). Furthermore, in both tissues, the total thiol content and SOD activity in the HTLV-1-infected group were lower than in the control group ( $P < 0.01$ ), and

Table 2: Pathological finding scores in the control and HTLV-1 infected groups

Pathological findings	Scores in groups	
	Control	HTLV-1-infected
Peribronchitis	0.00±0.00	1.57±0.29**
Bronchitis	0.00±0.00	0.57±0.29
Lymphoid follicles	0.14±0.14	0.14±0.14
Inflammation	1.00±0.00	1.14±0.14
Necrosis	0.00±0.00	0.00±0.00

The data is expressed as mean  $\pm$  SEM,  $n = 8$ . \*\* $P < 0.01$  compared to the control group

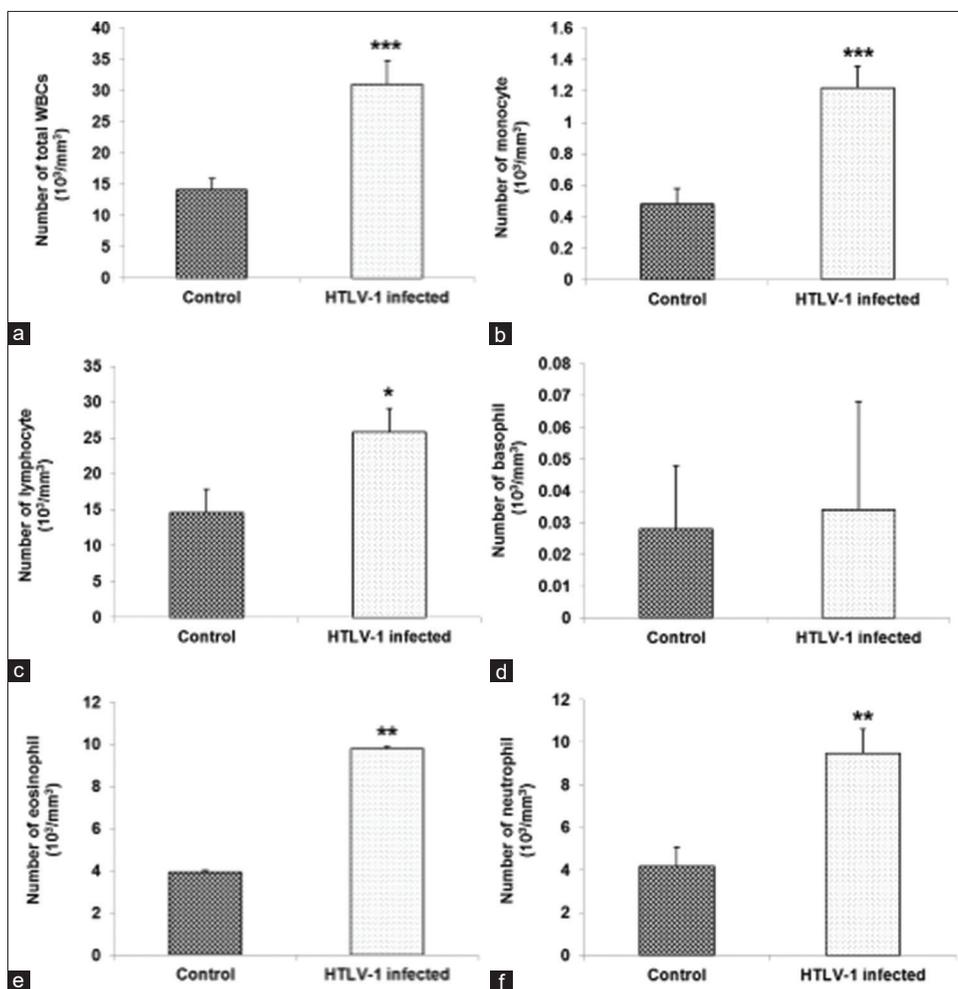


Figure 2: Total white blood cell number (count/mL of the blood) (a), number of monocytes (b), lymphocytes (c), basophils (d), eosinophils (e), and neutrophils (f) in the blood of the control and human T-cell leukemia virus type 1-infected groups. The data are expressed as mean  $\pm$  standard error of the mean,  $n = 8$ . \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$  compared to the control group

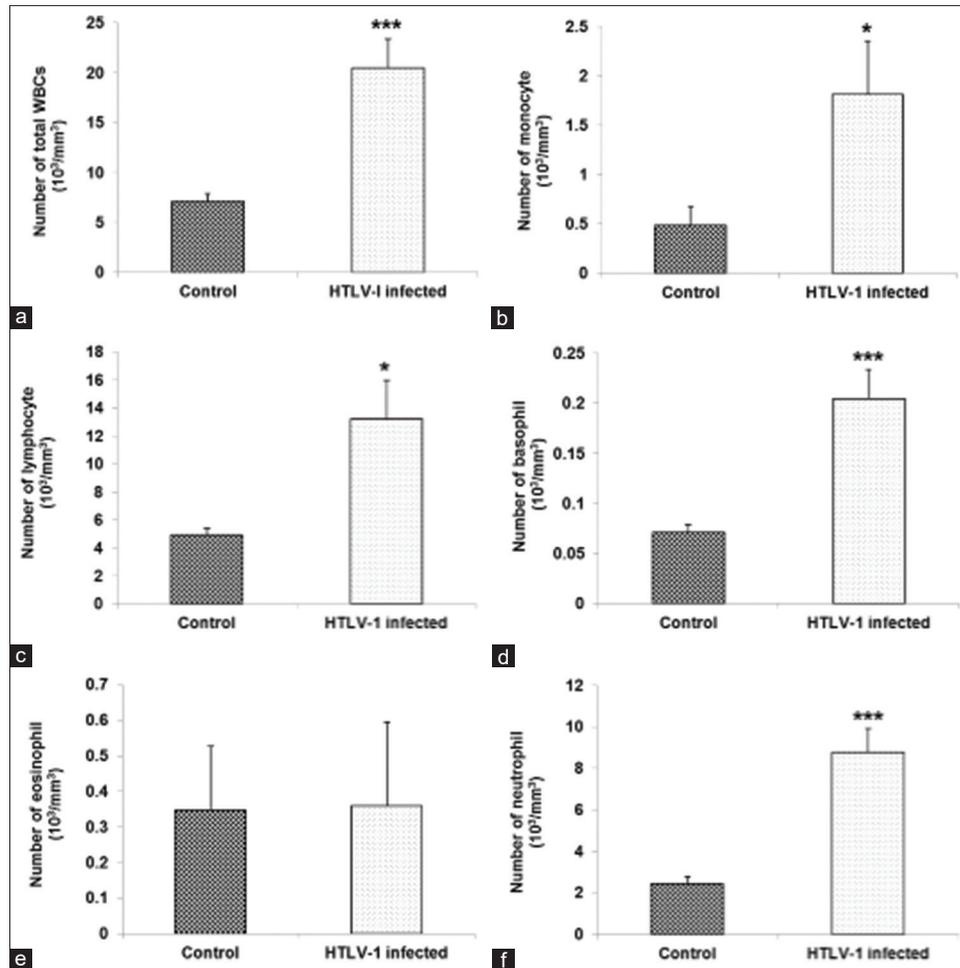


Figure 3: Total white blood cell number (count/mL of the bronchoalveolar lavage fluid) (a), number of monocytes (b), lymphocytes (c), basophils (d), eosinophils (e), and neutrophils (f) in the bronchoalveolar lavage fluid of the control and human T-cell leukemia virus type 1-infected groups. The data are expressed as mean  $\pm$  standard error of the mean,  $n = 8$ . \* $P < 0.05$  and \*\*\* $P < 0.001$  compared to the control group

Table 3: Level of MDA, total thiol, CAT and SOD activity in the BALF and lung of control and HTLV-1 infected groups

Tissue	Control	HTLV-I	Control	HTLV-I	Control	HTLV-I	Control	HTLV-I
	SOD U/g tissue		CAT U/g tissue		MDA nmol/g tissue		Thiol $\mu$ mol/g tissue	
Lung	5.19 $\pm$ 0.33	1.85 $\pm$ 0.5***	0.56 $\pm$ 0.00	0.58 $\pm$ 0.00	19.38 $\pm$ 2.56	34.68 $\pm$ 3.40**	0.92 $\pm$ 0.42	0.47 $\pm$ 0.05***
BALF	0.46 $\pm$ 0.03	0.33 $\pm$ 0.00**	0.04 $\pm$ 0.00	0.04 $\pm$ 0.00	1.36 $\pm$ 0.39	3.03 $\pm$ 0.29**	0.10 $\pm$ 0.00	0.07 $\pm$ 0.00***

The data is expressed as mean  $\pm$  SEM,  $n = 8$ . \*\* $P < 0.01$  and \*\*\* $P < 0.001$  compared to the control group. Malondialdehyde (MDA), Catalase (CAT), Superoxide dismutase (SOD) g tissue (ml) means 1 g of lung tissue or 1 ml of BALF

no significant change was seen in CAT activity in the lung tissue and BALF of the HTLV-1-infected group compared to the control.

### Discussion

The findings of oxidative stress analysis showed that MDA levels in the lung and BALF of the HTLV-1-infected group were higher than in the control group. Furthermore, the SOD activity and total thiol content in the lung tissue and BALF of the HTLV-1-infected group were lower compared to the control group. Pulmonary disorders are usually associated with increased oxidative stress. Foissac has demonstrated that

the serum total antioxidant capacity is decreased in patients with idiopathic pulmonary fibrosis.<sup>[23]</sup> Admittedly, viral infections can disturb the oxidant and antioxidant balance in the body.<sup>[24,25]</sup> Our previous study has demonstrated that infection with HTLV-1 increases oxidative stress in the mice brain tissues, including the cortex and hippocampus, via the destruction of some antioxidants such as total thiol, SOD, and CAT.<sup>[11]</sup> Similarly, Shomali has reported that the capacity of serum total antioxidant protective system is reduced in HTLV-1 carriers compared to normal people.<sup>[10]</sup> Oncoproteins, including Tax and c-Myc, possibly cause the promotion of complicated reactions in infected

cells and, consequently, the generation of ROS. Takahashi has introduced HTLV-1 Tax oncoprotein as a lymphocyte apoptosis inducer upon interfering with cellular oxidative balance.<sup>[26]</sup> Moreover, an *in vitro* study has shown that oxidative stress is induced by overexpressed c-Myc oncoprotein and can act as an intrinsic barrier against ATLL neoplastic disease.<sup>[12]</sup> Suresh has reported that a reduction of SOD activity and ROS accumulation in patients with the acquired immunodeficiency syndrome may have played a major role in the progress of HIV-associated tissue damages.<sup>[25]</sup> In an animal study, HIV transcriptional activator protein (Tat) has been recognized as the responsible factor in the production of deficient SOD.<sup>[27]</sup>

Furthermore, histopathological assessment in this study presented that the score of peribronchitis was higher in the HTLV-1-infected group compared to the control group; thus, HTLV-1 could induce peribronchitis injuries in the lungs. Several studies have reported that HTLV-1 may cause bronchiolitis<sup>[28,29]</sup> and/or bronchiectasis.<sup>[6]</sup> A multicenter cohort study has demonstrated that HTLV-1-infected patients have more pronounced biological changes in the lungs than asymptomatic carriers of HTLV-1.<sup>[29]</sup> Both animal and human studies have revealed that infection with other viruses such as HTLV-2,<sup>[29]</sup> HIV,<sup>[27]</sup> and adenoviruses<sup>[30]</sup> may amplify the risk of development of LIP and bronchiectasis. Ishikawa has reported that the pathology pattern of HABA and LIP seems to be uniform,<sup>[31]</sup> although other researchers have suggested differential patterns, including distributed micronodules associated with lymphocytic infiltration in HABA patients.<sup>[28,32]</sup> WBC counting results in the BALF of mice in this study showed that total WBC, the number of monocytes, lymphocytes, basophils, and neutrophils in the HTLV-1-infected group were significantly higher than in the control group. Itami<sup>[5]</sup> and Ishii<sup>[33]</sup> have shown that the pulmonary pathological alteration in an HTLV-1 carrier case includes diffuse multiple small nodular lesions and lymphocytic infiltration around the bronchioles. Injuries of the lung vascular endothelium induced by some viral infections<sup>[34]</sup> may cause leukocyte infiltration into the lung interstitial spaces and also BALF. Actually, by enhancing the secretion of pro-inflammatory cytokines,<sup>[35]</sup> as well as overproduction of ROS,<sup>[24]</sup> viruses progress lung and vascular damages. The level of oxidized proteins in BALF as an oxidative stress index positively correlates with inflammatory factors such as absolute counts of eosinophils and neutrophils as well as the serum levels of tumor necrosis factor-alpha, interleukin-1 (IL-1)  $\beta$ , and IL-8.<sup>[23]</sup> Accumulation of IL-2 receptor-expressed T-lymphocyte has been reported in the lungs of HABA patients.<sup>[14]</sup> Previous studies have indicated that the degree of Tax mRNA expression is a determinant factor in the severity of lung inflammation.<sup>[36,37]</sup> For instance, Higashiyama has reported that the percentage of the subpopulation of CD3+CD25 + lymphocyte in the lavage of carriers of HTLV-1 correlates with the level of Tax mRNA expression in pulmonary lesions.<sup>[36]</sup> Moreover, Seki has

suggested that the production of macrophage inflammatory peptide-1 $\alpha$  in HTLV-1-infected individuals with high Tax mRNA expression is higher than in HTLV-1-infected individuals with a low Tax mRNA expression.<sup>[37]</sup>

In the present study, the total WBC, the number of monocytes, lymphocytes, eosinophils, and neutrophils were significantly higher in the blood of the HTLV-1-infected group compared with the control group. Previous investigations have demonstrated that HTLV-1 infections are often associated with inflammatory cell activation and leukocytosis.<sup>[38]</sup> Moreover, in this study, eosinophilia was recognized in HTLV-1-infected mice. There are unpublished reports about severe lung diseases associated with eosinophilia in three of the HTLV-1-infected cases who visited the educational Ghaem Hospital (Iran) in 2017. Eosinophilia is an infection sign that plays a key role in the progress of bronchioloalveolar disorder.<sup>[39]</sup> Several studies have shown that infection with HTLV-1 is associated with eosinophilia.<sup>[40]</sup> In the study by Fukuoka, peripheral eosinophilia and raised serum IL-5 levels were not observed in all the HABA cases.<sup>[28]</sup> Therefore, Fukuoka has reported that eosinophilia is not a feature of DPB.<sup>[28]</sup> Similarly, Utsunomiya has declared that eosinophilia is not an ATLL prognostic sign.<sup>[33]</sup>

There was a nonsignificant increase in the other pathological changes, including bronchitis, lymphoid follicles, and necrosis in the HTLV-1-infected group as compared to the control group. These pathological changes may be manifested in a longer infection duration, and 2 months might not be enough for the occurrence of these pathological alterations.

## Conclusion

This study demonstrated that HTLV-1 causes peribronchitis, WBC accumulation, and oxidative stress in the pulmonary tissues. Therefore, HTLV-1 can facilitate the manifestation of bronchoalveolar remodeling by recruiting inflammatory cells. Particularly, HABAs may be the outcome of the expression of HTLV-1 oncogenes as well as HTLV-1-induced oxidative stress. Oxidative stress-induced by HTLV-1 infection can play a critical role in the infiltration of inflammatory cells into pulmonary tissues.

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## Conflicts of interest

There are no conflicts of interest.

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