

## Comparison of bleomycin-induced pulmonary apoptosis between NMRI mice and C57BL/6 mice

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### Abstract

Apoptosis has a critical role in the pathogenesis of bleomycin induced-pulmonary fibrosis. The severity of fibrosis varies among different strains of mice. Recent studies have indicated that expression of apoptotic regulatory genes may be specific in different cell types in various strains. In this study, bleomycin-induced pulmonary apoptosis in NMRI (Naval Medical Research Institute, USA) albino mice were compared with C57BL/6 black mice. Pulmonary fibrosis induced by single intratracheal administration of bleomycin (3 U/kg). Control mice were instilled with the same volume of saline. After 2 weeks, fibrotic responses were studied by biochemical measurement of collagen deposition and histological examination of pathological lung changes. Apoptosis was detected and quantitated by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay. Bleomycin significantly ( $P < 0.05$ ) increased lung collagen content and also induced fibrotic histological changes in both strains. Apoptosis was detected in the bronchiolar and alveolar epithelial cells after bleomycin instillation. TUNEL-positive alveolar epithelial cells in bleomycin-treated lungs of C57BL/6 and NMRI mice ( $19.5\% \pm 2.7$  and  $17\% \pm 2.0$ , respectively) were significantly ( $P < 0.05$ ) higher than that of saline-treated lungs ( $1.5\% \pm 0.5$ ) with no significant difference between two strains of mice ( $P > 0.05$ ). Despite some murine strain variation in the expression of apoptotic regulatory genes in bleomycin-induced pulmonary fibrosis, the results of the present study revealed no significant differences in alveolar epithelial apoptosis between NMRI and C57BL/6 black mice. However, these results confirm the role of apoptosis in the pathogenesis of pulmonary fibrosis and suitability of both strains as experimental models of lung fibrosis.

**Keywords:** Pulmonary fibrosis; Bleomycin; Apoptosis; NMRI mice; C57BL/6 mice

### INTRODUCTION

Pulmonary fibrosis is a compromising pathologic process resulting in organ failure in response to lung tissue damage. The patho-genesis and etiology of this life-threatening disease is still not well understood, but inflammation (1,2), apoptosis (3,4) and imbalance in extracellular matrix accumulation/remodeling have been implicated (5).

Apoptosis or programmed cell death is an important regulatory process for maintenance of tissue homeostasis. This pathway of cellular suicide controls cell numbers during physiological and various pathological processes, and

in a variety of mild injurious stimuli (heat, radiation, cytotoxic drugs, infection, etc.) (3,6). Apoptosis cascade may be initiated by extracellular or intracellular events resulting in fragmentation of the cell into apoptotic bodies which are engulfed by phagocytes (7). Apoptosis plays a critical role in the fibrotic lung diseases. Recent evidence suggests increased alveolar epithelial cell apoptosis, together with decreased apoptosis of myofibroblasts contribute in the initiation and progression of lung tissue injury into fibrosis (3,4,8). Various death receptors and ligands (TNF, Fas), death signals (reactive oxygen species, pro-inflammatory

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cytokines, chemo-kines, p53, etc) and regulator molecules of apoptosis (Bcl-2 family of proteins) have been identified in the pathogenesis of pulmonary fibrosis (9-11).

Bleomycin is an effective chemotherapeutic agent which its repeated systemic or high dose administration often leads to lung injury and fibrosis (12). Bleomycin-induced pulmonary fibrosis in animal models is very applicable for studying the cellular and molecular mechanisms of interstitial lung fibrosis (13). It is well known that sensitivities to profibrotic compounds such as bleomycin are strain-dependent, however the mechanisms of strain variation and the genetic basis of susceptibility to pulmonary fibrosis are largely unknown (14,15).

There are also limited studies about the genetic susceptibility to lung apoptosis. We previously reported that expression of apoptosis-regulatory genes, such as Bcl-2 and Bax may be affected by genetic variation in bleomycin-induced pulmonary fibrosis in two different strains of mice (16). In the present study, we compared bleomycin-induced pulmonary apoptosis between NMRI (Naval Medical Research Institute, USA) albino mice and C57BL/6 black mice (a fibrosis prone phenotype) for better understanding of the role of the genetic predispositions in pulmonary apoptosis.

## MATERIALS AND METHODS

### *Animals*

Female C57BL/6 black mice and NMRI albino mice, 8-10 weeks of age and 25-30 g of body weight, obtained from Pasteur Institute (Tehran, Iran) were used. Animals were housed in the standard laboratory cages at room temperature and ambient humidity. They had access to water and rodent laboratory chow *ad libitum*. The mice were acclimated to the laboratory conditions for at least 1 week prior to the commencement of the experiments. All experiments were carried out in accordance with international guidelines of the care and use of the laboratory animals.

### *Chemicals*

*In Situ* Apoptosis Detection Kit (TACS.XL Kit) was supplied by R&D Systems (USA). Ketamine was purchased from Rotexmedica Co. (Germany). Bleomycin hydrochloride was obtained from Nippon Kayaku Co. (Japan). L-

hydroxyproline and all other reagents for biochemical and pathological assays were purchased either from Merck (Germany) or Sigma Chemical Co. (St. Louis, MO).

### *Induction of pulmonary fibrosis*

To induce pulmonary fibrosis, animals were tracheostomized under anesthesia by intraperitoneal (i.p.) injection of 75 mg/kg of ketamine. Then bleomycin hydrochloride was administered as a single dose of 3 U/kg in 50  $\mu$ l of sterile saline intratracheally (i.t.) (12). Control mice received an equal volume of sterile saline intratracheally. Six animals were used in each control and experimental groups.

Two weeks after instillation of bleomycin, animals were sacrificed by a high dose of ketamine. Lungs were removed and then weighed. The right lung was carefully excised, and the wet weight was measured. Then, the lung was dried for 24 h at 60°C, and its dry weight was determined. The ratio between wet and dry lung weights is a measure of fluid content and edema formation in the lungs (17).

Samples of right lung were taken for biochemical analysis of collagen content. Samples of left lung were fixed, processed and 4- $\mu$ m-thick paraffin embedded sections were used for immuno-histochemical, haematoxylin-eosin (H&E) and Masson's trichrome staining and evaluation by light microscopy. Semiquantitative morpho-logical study of pathological changes in the lung sections was carried out to assess the severity of pulmonary fibrosis. These changes were graded according to the method described by Ashcroft and coworkers in a blinded fashion on a scale of 0 to 8 by examination of 10 randomly chosen regions per sample at a magnification of  $\times$ 100. These grades are as follows: grade 0=normal tissue; grade 1=minimal fibrous thickening of alveolar or bronchial walls; grade 3=moderate thickening of walls without obvious damage to the lung architecture; grade 5=increased fibrosis with definite damages to the lung structure and formation of fibrous bands or small fibrous masses; grade 7=severe distortion of structure and large fibrous areas; grade 8=total fibrous obliteration of the field (18).

### *Biochemical analysis*

Collagen content of lung tissues was estimated by determination of hydroxyproline

amount by colorimetric method (19). In brief, the dried right lungs were hydrolyzed in the presence of hydrochloric acid (6 N) for 8 h at 120°C. After homogenization and processing, 1 ml of chloramine-T solution (282 mg of chloramine-T, 2 ml of n-propanol, 2 ml of H<sub>2</sub>O, and 16 ml of citrate/acetate buffer) and then 1 ml of Ehrlich's solution (2.5 g of 4-dimethylaminobenzaldehyde, 9.3 ml of n-propanol, and 3.9 ml of 70% perchloric acid) were added to 50 µl of each sample. Finally absorbance of samples was read at 550 nm with a Unico UV-2100 spectrophotometer (United product, USA) (19).

### **Quantitation and detection of apoptosis**

Detection and quantitation of apoptosis was performed by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay (20). *In Situ* Apoptosis Detection Kit (TACS.XL Kit) was used to label the 3'-OH ends of fragmented DNA according to the manufacturer's protocol. Briefly, Paraffin-embedded lung tissue sections were rehydrated and treated with proteinase K solution for permeation. The slides were immersed in terminal deoxynucleotidyl transferase (TdT) labeling buffer. Then samples were covered with anti-bromodeoxyridine (anti-BrdU) and incubated with streptavidin-horse radish peroxidase (HRP) solution. Diaminobenzidine (DAB) was used as the chromogen and tissue sections were counterstained with methyl green. The slides were viewed in a blinded fashion. Cells containing fragmented nuclear chromatin, the characteristic of apoptosis exhibit a brown nuclear staining. For quantitation of apoptosis-positive epithelial cells, the numbers of positive cells within the surfaces of the alveolar walls were counted in a minimum of six randomly selected microscopic fields per lung section. The counts of positive nuclei per

field were expressed as a percentage of the total number of nuclei in the same microscopic field. Sections from each of at least five mice per treatment group were analyzed. For negative controls, unlabeled sample in which TdT enzyme was omitted was used for displaying the level of background labeling associated with non-specific binding of the streptavidin-HRP. This control did not have any brown staining. Another experimental negative control sample was normal or untreated tissues which have a small number of apoptotic cells.

### **Statistical analysis**

Data were presented as mean  $\pm$  S.E.M and analyzed using SPSS version 16, by unpaired Student's t-test and one-way ANOVA followed by Dunnett analysis. For grading analysis, the data were presented as semiquantitative grades and evaluated by the nonparametric Kruskal-Wallis method followed by Mann-Whitney tests.  $P < 0.05$  was considered statistically significant.

## **RESULTS**

### **Hydroxyproline assay**

Table 1 shows the results of lung collagen measurement by hydroxyproline assay in two strains of mice, 2 weeks after bleomycin instillation. Administration of bleomycin significantly increased hydroxyproline content by 48% and 44.5% in C57BL/6 and NMRI mice, respectively. However, there was no significant difference between the two strains of mice.

### **Lung weight**

Bleomycin significantly ( $P < 0.05$ ) increased wet/dry lung weight ratio, as an indicator of lung inflammation and edema formation, in both strains (Table 1).

**Table 1.** Fibrotic and inflammatory changes 2 weeks after bleomycin instillation (3 U/kg) in two strains of mice.

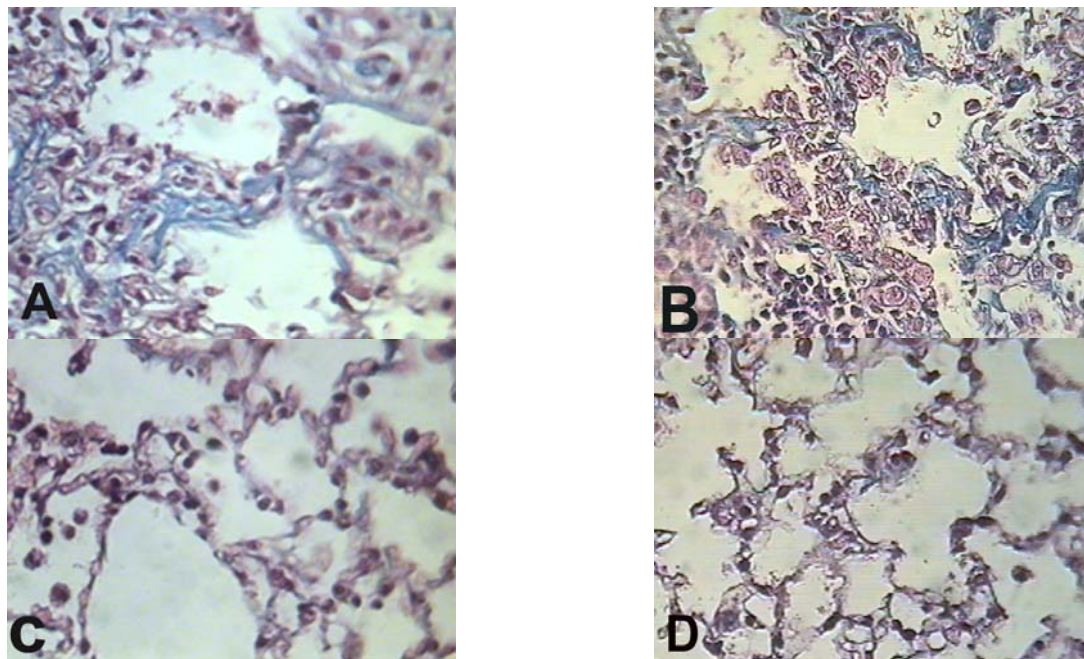
	Mice strain				P value
	C57BL/6		NMRI		
	Bleomycin	Control	Bleomycin	Control	
Hydroxyproline (µg/lung)	220.5 $\pm$ 25	136.6 $\pm$ 7.5	182.2 $\pm$ 13	125.5 $\pm$ 4	<0.05
Wet/dry lung weight ratio (mg)	4.9 $\pm$ 0.3	3.2 $\pm$ 0.2	5.5 $\pm$ 0.4	3.3 $\pm$ 0.3	<0.05

Values are means  $\pm$  SEM from 6 animals.  $P < 0.05$  compared to corresponding control.

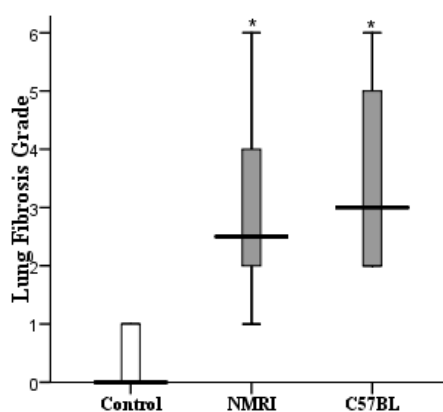
**Histological findings**

Two weeks after bleomycin instillation, there was pathological changes including thickening of alveolar walls, infiltration of inflammatory cells into the interstitium and increased amount of collagenous fibers in the interstitial areas in C57BL/6 and NMRI mice. No pathological change was observed in the

lungs of control animals (Fig. 1). The severity of changes varied from slight to moderate. According to the Ashcroft fibrotic score, the median grade was 3 for C57BL/6 and 2.5 for NMRI mice. The semiquantitative morphological changes of the lung tissue as fibrosis median grades are shown in Fig. 2.



**Fig. 1.** Representative Masson's trichrome histologic sections of lung tissues of mice. Thickening of the alveolar walls, increase in cellularity of alveolar septa and collagenous fibers (blue area) were observed in C57BL/6 (A), and NMRI mice (B), at 2 weeks after bleomycin instillation. There was no pathological change in the lung tissue of saline-treated C57BL/6 (C) and NMRI mice (D). Magnification:  $\times 400$ .



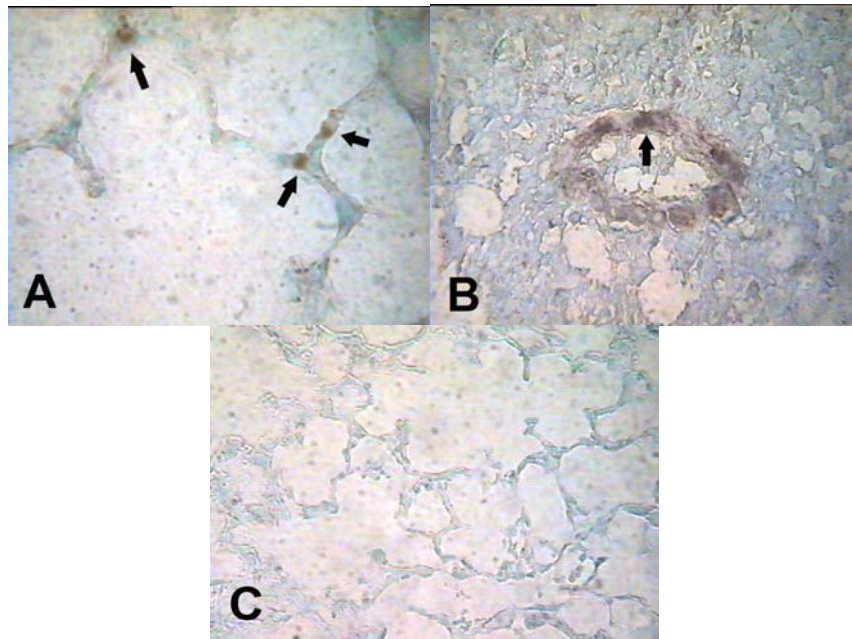
**Fig. 2.** Semiquantitative scoring of lung fibrosis at 2 weeks after bleomycin instillation (3 U/kg) in C57BL/6 and NMRI mice. Control mice received intratracheal saline. Thick lines represent the median of  $n = 6$ , boxes show the interquartile range and bars represent the maximum and minimum sample values. \*  $P < 0.05$  compared to control.

**Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay**

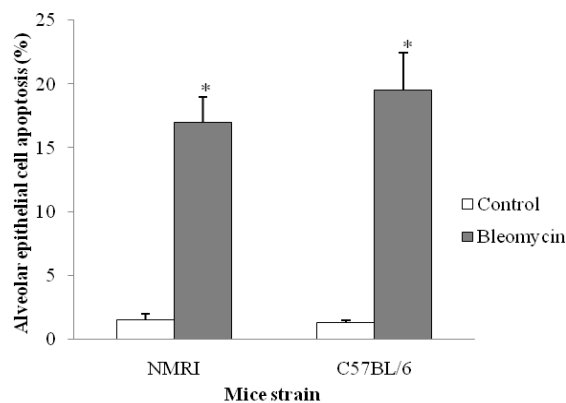
The positive signals for apoptosis (brown nuclear staining) were found in the bronchiolar and alveolar epithelial cells and also in a few endothelial cells of blood vessels 2 weeks after bleomycin instillation (Fig. 3 A, B). In the control group treated with saline instillation, positive signals were observed only in a few

alveolar epithelial cells (Fig. 3 C).

Quantitation of apoptosis revealed that bleomycin instillation increased the abundance of TUNEL-positive alveolar epithelial cells to  $19.5\% \pm 2.7$  in C57BL/6 and  $17\% \pm 2$  in NMRI mice. TUNEL-positive alveolar epithelial cells in bleomycin-treated lungs were significantly ( $P < 0.05$ ) more than that of saline-treated lungs ( $1.5\% \pm 0.5$ ) (Fig. 4).



**Fig. 3.** Representative staining results of apoptosis detection by TUNEL assay in lung tissues of mice. The positive signals for TUNEL are observed in alveolar (A) and bronchiolar epithelial cells (B), 2 weeks after exposure to intratracheal bleomycin (3 U/kg). There are no signals in alveolar epithelial cells in control mice treated with intratracheal saline (C). Magnifications:  $\times 1000$  (A),  $\times 400$  (B, C).



**Fig. 4.** Percentage of alveolar epithelial cells apoptosis in lung tissues of mice by TUNEL assay. C57BL/6 and NMRI mice were exposed to intratracheal bleomycin (3 U/kg). Control mice received intratracheal saline. After 2 weeks, lung sections were prepared and labeled by TUNEL assay. Labeling was quantitated in cells within the alveolar surfaces. Data are presented as mean  $\pm$  SEM of  $n = 5$ . \* =  $P < 0.05$  compared o corresponding control.

## DISCUSSION

Strain variation in fibrotic responses to bleomycin has been reported among several different strains of mice (14,15). Various levels of key mediators in fibrotic disorders such as chemokines, growth factors and tissue inhibitor of metalloproteinase, likewise DNA scission and matrix protein mRNA levels may be involved in strain differences in susceptibility to fibrogenic stimuli (21-23). Investigation of genetic basis of strain variation have revealed differentially expression of pulmonary genes including those of heparin binding and extracellular matrix deposition pathways (15), genes involved in oxidative stress response in apoptosis and in immune regulation (24). The NMRI mouse which originally derived from Swiss mice is a largely used animal in many fields of pharmacology and toxicology (25). We previously established bleomycin-induced pulmonary fibrosis and described its time-courses in this strain of mice (12). Our further study on apoptosis-regulatory genes, revealed some differences in expression of Bcl-2 and Bax in myofibroblasts, neutrophils and lymphocytes between C57BL/6 and NMRI mice (16).

In this study, the fibrotic and inflammatory changes were observed at two weeks after bleomycin instillation in both strains of mice with similar ranges. Fibrosis median grades were 3 and 2.5 in lung tissues of C57BL/6 and NMRI mice, respectively. Other studies have also reported similar results for pathology index score in C57BL/6 mice (26,27). The result of present study also showed apoptotic changes in the bronchiolar and alveolar epithelial cells at two weeks after administration of bleomycin intratracheally, although there was not any significant difference between two strains of mice. Li and his coworkers also reported similar results from apoptosis of alveolar epithelial cells (17.5%  $\pm$  1) by using *in situ* end labeling (ISEL) of fragmented DNA at 14 days after intratracheal bleomycin (21). Similar to our result, Hagimoto and his coworkers also reported the persistent appearance of TUNEL-positive signals in alveolar epithelial cells over

a period of 14 days (28) whereas Aoshiba K and his coworkers reported that the positive TUNEL signals in bronchial and alveolar epithelial cell disappeared by 10 days after bleomycin instillation (29).

TUNEL technique which was used in this research has been reported to be much more sensitive in detecting apoptotic cells, especially epithelial cells and at an early stage, in comparison with morphological scoring of cells or flow cytometric analysis, although some false-negatives data may be found in this technique. The results of our previous study showed overexpression of Bax protein in alveolar epithelial cells of both strains which suggests increased alveolar epithelial cells apoptosis in agreement with result of the present study. We also had found overexpression of Bcl-2 protein in myofibroblasts and neutrophils of NMRI mice lung tissue and also a decrease in Bax expression in myofibroblasts and lymphocytes of the lung tissues of C57BL/6 mice as well as in lymphocytes of NMRI mice after bleomycin instillation. These findings suggest decreased apoptosis of myofibroblasts, neutrophils and lymphocytes. Unfortunately, we could not find any apoptotic changes in other cells except bronchiolar and alveolar epithelial cells and also in a few endothelial cells in the lung tissue by TUNEL assay. Other techniques such as flow cytometry may be more appropriate for detecting apoptosis in other cells including fibroblasts (30).

In this research, the apoptotic changes were similar between two strains of mice. However, several studies have revealed differences in severity of apoptosis between susceptible murine strains (such as C57BL/6) and resistant strains (such as BALB/c) (31, 14). Pottier and his coworkers have reported more pronounced apoptosis after bleomycin administration in C57BL/6 mice. In their report, caspase-3 activity as a marker of execution-phase of apoptosis and caspase-8 activity as a marker of receptor-mediated apoptosis have been more obvious in C57BL/6 strain than BALB/c strain while caspase-9 activity as an initiator caspase in apoptosis has been similar in two strains (31).

Different biological characteristics of strains of mice represent variation between individual humans and could be helpful for further understanding of human disease. However in this study, variation in fibrotic and apoptotic changes was not observed between two strains of mice and similarity of lung responses in NMRI with C57BL/6 mice (as a sensitive strain to bleomycin) makes NMRI mice strain suitable as an experimental model for interstitial lung disease.

### CONCLUSION

In conclusion, findings of this study confirm the suitability of NMRI mice strain as an experimental model for lung fibrosis and apoptosis, and also highlight the essential role of increased alveolar epithelial cells apoptosis in the pathogenesis of pulmonary fibrosis. Novel therapies based on the regulation of apoptosis may have beneficial effect in the treatment of patients with interstitial lung disease.

### ACKNOWLEDGMENT

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