

Evaluation of the Effects of Nicotinamide on the Bleomycin-induced Pulmonary Fibrosis in Rat

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Abstract: Fibrosis is the abnormal production of collagen fibers following tissue damage. This accounts for the formation of scar tissue. Lung fibrosis is one of the typical ways in which the lung reacts to damaging stimuli. In this study we tried to investigate the involving phenomena during the process of bleomycin-induced fibrosis in murine lung. We have evaluated this process at three levels, namely, at (1) cellular level (cells with contraction activity of pulmonary tissue), (2) intercellular signaling agents (e.g., IL-8, TNF- α , TGF- β), tissue index factors (collagen, hydroxyproline) and some inorganic elements which may hypothetically be engaged as efficient enzymatic cofactors (e.g., copper), (3) pathophysiological or exaggerated physiological processes (inflammation and fibrosis). The pharmacological agent which have been selected for evaluating in this study is Nicotinamide, which their selection rationale will be discussed in detail separately in the discussion section. Bleomycin-induced pulmonary fibrosis is a widely used animal model for lung injury and fibrosis. After single dose instillation of intratracheal bleomycin, the fibrotic responses were studied by biochemical measurement of collagen deposition and analysis of pathological lung changes in different treatment groups. The results of this study showed that administrated agents in different doses, had satisfactorily healing effects on fibrosis process, ranging from good to moderate, through significant decreasing in lung collagen content ($p < 0.05$).

Key words: Bleomycin, L- Hydroxyproline, nicotinamide, pulmonary fibrosis, rat

INTRODUCTION

From man's earliest attempts to manufacture tools and cloths and construct buildings, he has faced health risks from inhalable substances or dusts in the workplace. With sophistication of life and creation of villages, cities and societies, such risks became more serious. The earliest recorded acknowledgement of this problem comes from the time of Hippocrates, (377-460 B.C), the father of medicine. He knew that inhaled dust could affect the health and stated: "The metal miner is a man who breathes with difficulty". Georgius Agricola, in his book, 'De Re Metalica' (1556), wrote about dusts and suggested that they ulcerate the lung and cause consumption. He described several methods for ventilation of mines to reduce the risk of lung disease (Holt, 1987). Fibrosis is the abnormal production of collagen fibers following tissue damage. This accounts for the formation of scar tissue. Lung fibrosis is one of the typical ways in which the lung reacts to damaging stimuli. Historically, idiopathic pulmonary fibrosis was thought to be a

deleterious consequence of persistent lung inflammation that followed an unknown insult. However, the growing recognition that foci of proliferating fibroblasts are more prominent than evidence of inflammation in lung tissue (Kuhn, 1989), as well as the lack of efficacy of anti-inflammatory drugs in the majority of affected patients, has led to a focus on the fibrotic pathway itself (Selman *et al.*, 2001). At first blush, the article by Kolb *et al.* (2001) in this issue of the *JCI* (Kolb *et al.*, 2001) might suggest that we may have discounted inflammation too hastily. The authors show that treatment of rats with a single dose of a recombinant adenovirus encoding the cytokine IL-1 β , a central regulator of acute inflammation (Dinarello, 1996) leads to a progressive form of pulmonary fibrosis that continues over at least 60 days. Importantly, this progression is not due to any persistent effects of IL-1 β itself, since, as expected with adenovirus infection, IL-1 β expression is only increased transiently, returning to near base-line values by 14 days after infection. In contrast, pulmonary fibrosis (as determined by increases in lung hydroxyproline content) is not

apparent until day 21 and dramatically increases thereafter. Thus, IL-1 β can be added to a short list of agents, including the cytotoxic drug bleomycin (Jones and Reeve, 1978), ligands of the Fas death receptor (Hagimoto, 1997), and the cytokine TGF- β 1 (Sime *et al.*, 1997), that can bypass the normal repair process and initiate a self-perpetuating cycle of pulmonary fibrosis. Should the article by Kolb *et al.* (2001) (Giri *et al.*, 1993) rekindle enthusiasm for focusing on acute inflammation in our efforts to better understand the mechanisms underlying diseases characterized by progressive pulmonary fibrosis.

Bleomycin is commonly used as a part of the cytostatic treatment of several tumor types, such as germ-cell tumors, lymphomas, and Kaposi's squamous cell carcinomas of head and neck. The application of bleomycin is featured by the occurrence of some fatal side effects. Pulmonary toxicity is the most serious side effect of BLM. In lung, the toxicity of BLM involved inflammation and fibrosis. Bleomycin-induced pulmonary fibrosis is an animal model for lung injury and fibrosis (Nuno *et al.*, 1998). Pulmonary fibrosis, idiopathic or otherwise, is commonly progressive and essentially untreatable, with a fatal outcome (Coker and Laurent, 1997; Zhang and Phan, 1996). There are five million people worldwide that are affected by this disease. In the United States there are over 200,000 patients with Pulmonary Fibrosis. As a consequence of misdiagnosis the actual numbers may be significantly higher. Of these more than 40,000 expire annually. This is the same as the people who die from Breast Cancer. Typically, patients are in their forties and fifties when diagnosed. However, diagnoses have ranged from age seven to the eighties.

The pathogenesis of pulmonary fibrosis remains incompletely understood. Studies of associated inflammation have led to the discovery of a number of cytokines and chemokines that are found to be important either directly or indirectly for the fibrotic process. So, it is clear from a rather wide body of work that the underlying mechanism involves dysregulation and overproduction of certain cytokines (Smith *et al.*, 1995; Hogaboam *et al.*, 1998). However, the importance of inflammation in pulmonary fibrosis is unclear, and at the time of diagnosis the inflammatory component is variable and usually not responsive to anti-inflammatory therapeutic agents. A central mechanism appears to involve continued overproduction of TGF- β (transforming growth factor), which could in turn increase the production, activity, or both of CTGF (connective-tissue growth factor) (Khalil and Greenberg, 1991; Border and Noble, 1994; Lasky *et al.*, 1998; Brigstock, 1999; Pittet *et al.*, 2001). In addition to promoting myofibroblast differentiation, transforming growth factor- β (especially TGF- β 1) provides protection against apoptosis. Thus, this well-known fibrogenic cytokine is important both for the emergence of the myofibroblast and its survival against

apoptotic stimuli. This is consistent with the critical importance of this cytokine in diverse models of fibrosis in various tissues (Denis, 1994; Allen *et al.*, 1999; Pittet *et al.*, 2001; Kuwano *et al.*, 2005). Considering the abovementioned short introduction, we practically wanted to investigate the involving phenomena during the process of bleomycin-induced fibrosis in murine lung. We have evaluated this process at three levels, namely, at (1) cellular level (cells with contraction activity of pulmonary tissue), (2) intercellular signaling agents (e. g., IL-8, TNF- α , TGF- β), tissue index factors (collagen, hydroxyproline) and some inorganic elements which may hypothetically be engaged as efficient enzymatic cofactors (e.g., copper), (3) pathophysiological or exaggerated physiological processes (inflammation and fibrosis). The pharmacological agent which have been selected for evaluating in this study is Nicotinamide, which their selection rationale will be discussed in detail separately in the discussion section.

MATERIALS AND METHODS

Chemicals: Bleomycin (Nippon Kayaku Co. Ltd, Japan), Ketamine (Rotexmedica Co., Germany), Nicotinamide and L-hydroxyproline (the latter three from Sigma Chemical Co, England) were used. All other analytical grade reagents for histology and biochemical assays were bought either from Merck (Germany) or Sigma Chemical Co. (England).

Animals: Male Sprague-Dawley rats weighing 180-200 g were used during the study. Animals were held in an air-conditioned room with 12 h light cycle at 21-24°C and 45-55% humidity, and they were fed on standard laboratory chow and tap water *ad libitum*.

Induction of pulmonary fibrosis by bleomycin: This study was conducted in the research laboratory of the department of pharmacology of School of Pharmacy, Jondishapour University of Medical Sciences, Ahwaz, Iran, in 2008. The rats, according to the method of Schraufnagel *et al.* (1986) were anaesthetized with ether, and then they were placed on a slanted board (20° from vertical) hanging from their upper incisors. Bleomycin (7.5 IU/kg) was delivered via the mouth into the trachea with a modified syringe needle in a volume of 1 ml/kg body weight. So the animals received an intratracheal injection of bleomycin (7.5 IU/kg) in saline solution (Schraufnagel *et al.*, 1986). The rats were rotated immediately after receiving bleomycin to ensure that good drug distribution occurred in the lung. After recovery from anesthesia, the rats were returned to their cages and allowed food and water as normal. Control rats received an intratracheal instillation of the same volume of sterile saline. Their food intake, respiration, and activity were observed every day (Szapiel *et al.*, 1979).

Drugs treatment: The animals were divided into the following groups, (n = 6 rats). 1. A group received only bleomycin as “positive control”. 2. The rats in this group received vehicle (normal saline) as “negative control”. 3. “Treatment-N1 group” received daily nicotinamide 50 mg/kg/day, 7 days before and 4 weeks after administering single-dose bleomycin (Melo *et al.*, 2000). 4. “Treatment-N2 group” received daily nicotinamide 300 mg/kg/day, 7 days before and 4 weeks after administering single-dose bleomycin (Nuno *et al.*, 1998). All the administration routes were as intraperitoneal (IP) and the vehicle in all solutions was distilled water. We also investigated the effects of the drugs and the vehicle (without bleomycin) as sham groups. 5. Nicotinamide 7 mg/kg/day plus the vehicle for 5 weeks as “sham N”.

Determination of collagen and hydroxyproline content of lung tissue: Hydroxyproline content of lung tissue was determined by colorimetric method as described by Edwards and O’Brien (Pittet *et al.*, 2001). Total content of tissue collagen was calculated with the assumption that 12.5% of collagen is constituted of hydroxyproline. Hydroxyproline is extracted from collagen and can be oxidized to pyrrole by chloramine-T, and then it can produce color with *para*-dimethylbenzaldehyde. Tissue samples will be homogenized and processed according to the earlier discussed method. The absorbance will be measured at 500 nm to determine hydroxyproline content. The hydroxyproline value is divided by 0.125 to express collagen content of tissue (mg/g tissue) (Khalil and Greenberg, 1991)

Histological examination: Lung tissue was fixed by 10% neutral formalin solution for paraffin slides and sectioned at approximately 5- μ m thickness. Tissue affixed to a glass slide deparaffinised, rehydrated and counterstained with hematoxylin and eosin (H&E) or Masson’s trichrome. The slides were examined by light microscopy and photographed (Khalil and Greenberg, 1991).

Statistical analysis: Data will be presented as the mean \pm SEM. For determination of the significant differences, statistical analyses will be performed using student’s t-test or one-way ANOVA and also Kruskal-Wallis by SPSS software. Values of p<0.05 will be regard as statistically significant.

RESULTS

Body and lung weights: Generally, there was a significant body weight loss in the groups, which received bleomycin. But the treatment groups had moderate weight loss (Fig. 1). Total wet lung weight was measured as an indicator of lung inflammation due to Bleomycin (7.5 IU/kg).

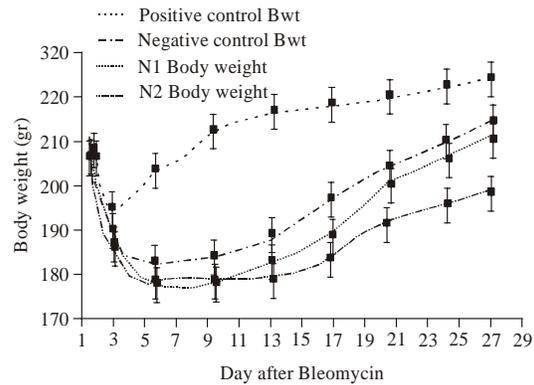


Fig. 1: The effect of bleomycin and/or nicotinamide on body weight of the animals during the study. Data are presented as mean \pm SEM of n = 6. Positive control: bleomycin (7.5 IU/kg single dose); negative control : normal saline; N1: nicotinamide 50 mg/kg/day; N2: nicotinamide 300 mg/kg/day, Bwt: Body Weight.

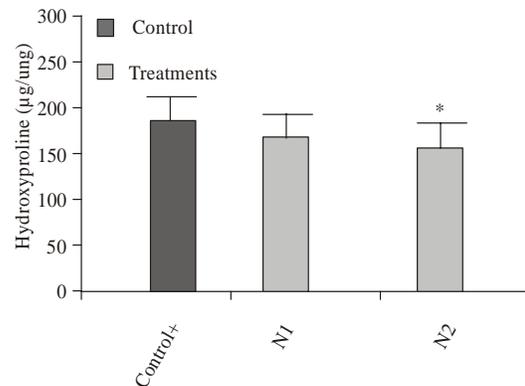


Fig. 2: The effect of bleomycin on lung hydroxyproline content in the studied animals. Hydroxyproline content of lung tissue of mice was measured and normalized to micrograms per lung. Data are presented as mean \pm SEM of n = 6; *: p<0.05 versus control. Positive control: bleomycin (7.5 IU/kg single dose); N1: nicotinamide 50 mg/kg/day; N2: nicotinamide 300 mg/kg/day.

Hydroxyproline content of lung: To determine and analysis of lung fibrosis, lung collagen was measured as hydroxyproline content after euthanizing animals at the end of the experimental course. Our findings showed that administration of bleomycin (7.5 IU/kg) significantly increased (p<0.001) hydroxyproline level in positive control group. Other groups had different results which have been summarized in the Fig. 2.

Histology: Morphological examination of lungs was carried out at the end of the study course (Fig. 3). Histological analysis showed that the treatment groups had less pathological changes in lung in comparison to the

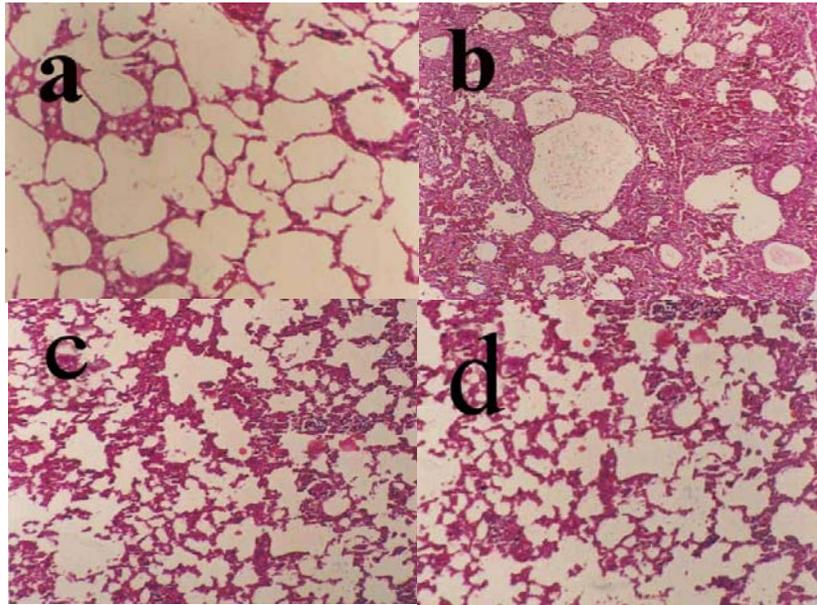


Fig. 3: a-d: Hematoxylin-eosin histological sections of lung tissue of different groups (magnifications $\times 100$). (a) normal lung tissue of saline treated rat (control negative group); (b) there is an increase in cellularity of alveolar septal and intraalveolar fibrosis with collagenous bands accompanying great septal thickness and diffuse damage to lung architecture are observed (control positive group); (c) interstitial fibrosis with scattered septal thickness but slight infiltration of lymphocytes in comparison to the previous section (b) (nicotinamide group); (d) decreased fibrosis although it can be seen focally somewhere (group N1); N1: nicotinamide 50 mg/kg/day; N2: nicotinamide 300 mg/kg/day.

positive control group. However, in different treatment groups, the severity of changes varied from slight to moderate. The comparative analysis of the lung tissue of the different groups has been presented in Fig. 3(a-d), in detail.

DISCUSSION

Bleomycin, when given to cancer patients, may produce pulmonary fibrosis in about 3%. On the basis of these observations, a mouse model of pulmonary fibrosis has been established in which intratracheal instillation of bleomycin uniformly produces pulmonary fibrosis (Nuno *et al.*, 1998). This model is thought to be a model of human pulmonary fibrosis (Pittet *et al.*, 2001).

Severe lung injury induces excessive cell death. Maintaining normal function and repair of parenchymal cells is the key to improving the prognosis of patients. Excessive cell death of parenchymal cells means irreversible tissue damage and may lead to pulmonary fibrosis.

In this study the pathological changes in the lung tissue caused by bleomycin, were consistent with the findings of the other studies (Khalil and Greenberg, 1991;

Hübner *et al.*, 2008). The main features observed were intra-alveolar amorphous and cellular debris, hyaline membranes, interstitial edema and fibrosis, atypical proliferation of alveolar epithelial cells and collapse of alveoli.

Bleomycin affects on the contractility of lung tissue strips and the lung collagen contents. Additionally the cytokine levels are affected by bleomycin administration. The underlying mechanisms rest on physiology and pathophysiology of the intervening factors. The lung Extracellular Matrix (ECM) consists of collagen and elastic fibers interspersed with structural glycoproteins and proteoglycans (PGs). PGs influence lung tissue mechanical properties, and through their interactions with various macromolecules, contribute to a variety of biological functions. Changes in ECM synthesis and degradation play a part not only in physiological processes such as development, growth and aging but also in wound healing, inflammation and fibrosis (Couch, 1990).

It is worth to note that, bleomycin-induced pulmonary fibrosis is an inflammatory interstitial lung disease characterized by excessive accumulation of fibroblasts and ECM molecules, including PGs, in the

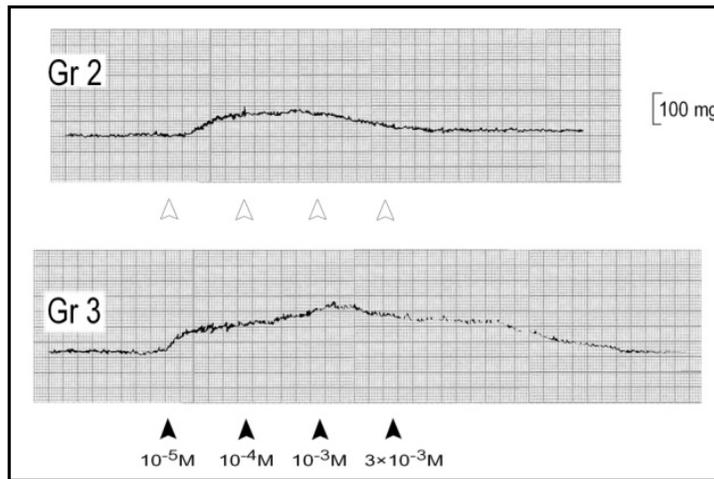


Fig. 4: Representative traces showing responses of rat lung strips to cumulative doses of acetylcholine. In this figure the responses of group 3 (Gr 3: “Treatment-N1 group” received daily nicotinamide 50 mg/kg/day, 7 days before and 4 weeks after administering a single-dose bleomycin (7.5 IU/kg)). has been demonstrated in comparison to negative control group (Gr 2: received vehicle (normal saline) as “negative control”). Each division represents one minute. ▲ : Administration of drug.

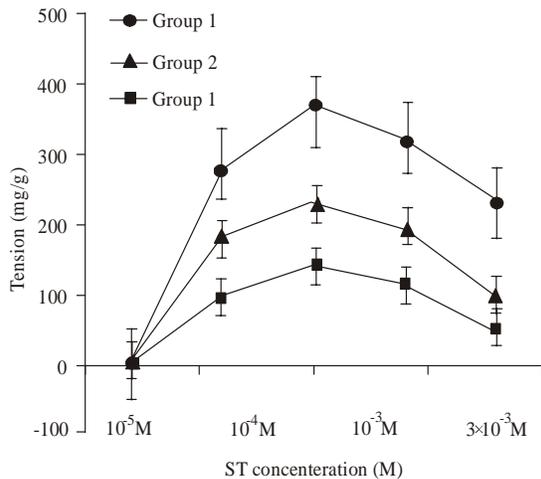


Fig. 5: The effects of sodium tungstate on contractions of positive control (Gr 1) and negative control (Gr 2: received vehicle (normal saline) as “negative control”). rat lung strip preparations in comparison to group 3 (Gr 3: “Treatment-N1 group” received daily nicotinamide 50 mg/kg/day, 7 days before and 4 weeks after administering a single-dose bleomycin (7.5 IU/kg)). Each point represents the mean±SEM (n = 6); between groups: * p<0.05 and ** p<0.01, Mann-Whitney U-test.

intraluminal and interstitial compartments of the lung. Evidence from human studies and animal models indicates that TGF-β1 plays a pivotal role in mediating pathophysiological changes in fibrotic diseases (De Vos

et al., 2008). TGF-β1 stimulates fibroblasts to synthesize large amounts of ECM proteins. TGF-β1 levels are upregulated in patients with pulmonary fibrosis and also in bleomycin-induced lung fibrosis in rats (Fig. 4 and 5).

Fibroblasts participate in inflammatory responses and wound repair through their ability to release cytokines, secrete ECM proteins and through cell-cell interactions with other inflammatory cells such as macrophages. At sites of injury and wound repair, fibroblasts have been shown to migrate from different anatomic sites and transform into a less proliferative but more contractile and collagen synthetic phenotype. It has been reported that lung fibroblasts cultured from bleomycin-induced fibrotic rat lungs produced more collagen than normal lung fibroblasts in culture. Furthermore, Raghu *et al.* demonstrated that TGF-β1 stimulated collagen production and collagen mRNA levels in fibroblasts derived from normal and fibrotic human lungs (De Vos *et al.*, 2008).

Nicotinamide, also known as niacinamide, and niacin have identical vitamin activities (i.e., they both prevent development of the vitamin B3-deficiency condition, pellagra), and also they have very different pharmacological activities (Tesfaigzi *et al.*, 2004). The structure of niacinamide consists of a pyridine ring with an amide group in position three. Niacinamide is a component of Nicotinamide Adenine Dinucleotide (NAD), also known as coenzyme I, and nicotinamide adenine dinucleotide phosphate (NADP), also known as coenzyme II. These coenzymes are involved in many intracellular oxidation-reduction reactions. They

participate in hydrogen transfer reactions, functioning as hydride ion carriers of biological systems (Sanchez *et al.*, 1996).

It has been shown that Lung Nicotinamide Adenine Dinucleotide (NAD) depletion accompanies bleomycin-induced lung fibrosis in the hamster and that treatment with niacin (NA), a precursor of NAD, was found to attenuate lung fibrosis caused by this agent (Segura-Valdez *et al.*, 2000). The histopathological studies revealed that niacin treatment attenuated bleomycin-induced thickened alveolar septa, foci of fibrotic consolidation, and accumulations of inflammatory cells in the parenchyma and air spaces (Majo *et al.*, 2001). The ability of niacin to attenuate BLM-induced lung fibrosis in hamsters suggests that it may have potential as an antifibrotic agent in humans (Yokohori *et al.*, 2004). Some studies also suggest that Nicotinamide induces apoptosis in myofibroblasts in pulmonary fibrosis (Majo *et al.*, 2001).

As it was found out during review of the literature, the previous works have been done on hamsters, we studied the protective effects of nicotinamide on rats as an animal model for human. Before this study, the effects of nicotinamide had not been evaluated on bleomycin-induced pulmonary fibrosis in the rat.

Our results confirm the effectiveness of nicotinamide but only in higher doses. According to the results of our histological and immunological tests, there was no significant difference between lower dose of nicotinamide and the results of positive control group. Histological studies showed interstitial fibrosis with scattered septal thickness and infiltration of lymphocytes approximately similar to the positive control. These results show that there are no promising changes in comparison to the positive control group. The results of the ELISA also showed no significant differences in comparison with the positive control group (TGF-beta, TNF-alpha and IL-8, $p > 0.05$).

However, the results of higher dose of nicotinamide has had more promising results, as reported in previously mentioned work (Yokohori *et al.*, 2004). The histological section of lung tissue in this group showed scattered septal thickness and slight infiltration of lymphocytes in comparison to the positive control group. The results of ELISA tests also showed a significant difference of high dose nicotinamide in comparison to the positive control group (TGF-beta, TNF-alpha and IL-8, $p < 0.05$).

A similar study may be conducted in more precise ranges of different doses of nicotinamide. But now, according to these results, and considering that we duplicated our ELISA tests, there may be an interesting reason for this difference! It may be due to the role of

nicotinamide in the physiological processes. As we mentioned above, this agent is a physiologically active agent and essential substrate for normal processes of the body. So it seems that when we administer this agent in lower doses it acts its physiological roles, but in the higher doses its pharmacological effects begins to appear and we can see its protective effects on pulmonary fibrosis, only in the higher doses.

A adjusting the doses of nicotinamide may be a weak point for using this agent in the clinical conditions. For this purpose, it is necessary to monitoring the patient for his or her serum level of nicotinamide. On the other hand, some reports about toxic effects of this substrate in higher doses are available (Segura-Valdez *et al.*, 2000; Majo *et al.*, 2001). Finally we suggest again nicotinamide is a reasonable candidate for clinical application or other experimental studies in lung fibrosis.

ACKNOWLEDGMENT

The authors wish to thank Dr. Ardeshir Arzi, Mrs. Engin. Nazari and Mr. Mehdi Sameie for their assistance with this study. This research was supported by a grant from the research council of Ahvaz Jondishapour University of Medical Sciences.

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