



Protective Effect of Grape Seed Extract against the Fibrogenic Effect of Bleomycin in Rat Lung

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Abstract

Many studies have been performed for treatment or prevention of pulmonary fibrosis. However, no effective treatment has been found yet. The aim of this study was to investigate the effect of grape seed extract on bleomycin-induced lung fibrosis in rat. Hydroalcoholic extract of grape seed (*Vitis vinifera*) was prepared using maceration method. NMRI rats weighing 250-300 g were given single intratracheal instillation of bleomycin (7.5 IU/kg=5 mg/kg) or saline. The experimental groups were treated with a single dose of bleomycin followed by different doses of oral grape seed extract (100, 200, 400 mg/kg/day) or vitamin E (20 IU/kg) for two weeks, and then the animals were sacrificed and lungs were removed for histology and biochemical investigation. Histopathological examination of bleomycin-treated animals showed that bleomycin caused marked alveolar thickening associated with fibroblasts and myofibroblasts proliferation and collagen production in interstitial tissue leading to pulmonary fibrosis. Administration of grape seed extract reduced fibrotic damages in lung tissue in a dose-dependent manner. The effect of grape seed was comparable to that of vitamin E. Collagen and hydroxyproline contents of lung tissue were determined using spectrophotometric method. Lung weight, hydroxyproline and collagen amounts in bleomycin treated animals were significantly higher than in normal, vitamin E and grape seed treated groups. From this study, it can be concluded that grape seed extract may be able to diminish the fibrogenic effects of bleomycin on lung. This effect of grape seed can be attributed to active ingredients of the plant with anti-oxidant properties.

Keywords: Bleomycin; Grape seed; Pulmonary fibrosis; Vitamin E.

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1. Introduction

Interstitial pulmonary fibrosis is

characterized by an altered cellular composition of the alveolar region with excessive deposition of collagen. Typical features in this disease include dyspnea, diffuse interstitial infiltrates, progressive lung fibrosis and poor prognosis. The etiology of this

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disease is unknown; however, lung inflammation is a major underlying component of a wide variety of pulmonary fibroproliferative disorders [1]. Reactive oxygen species (ROS), such as superoxide, hydrogen peroxide, peroxyxynitrite and hydroxyl radical, are major mediators of lung inflammatory processes [2].

Many xenobiotics that stimulate the overproduction of ROS, such as bleomycin, paraquat, silica and hexavalent chromium [3], are capable of producing lung fibrosis. Yet, the direct linkage of ROS formation and pulmonary fibrosis has not been firmly established [4]. One of the clinically relevant causative agents of pulmonary fibrosis is the antineoplastic agent bleomycin, which is widely used in animal models to cause oxidant-induced inflammatory and fibrotic lesions in the lung [5].

Free radicals have been implicated in over a hundred disease conditions in humans, including arthritis, hemorrhagic shock, atherosclerosis, advancing age, ischemia and reperfusion injury of many organs, Alzheimer and Parkinson's disease, gastrointestinal dysfunctions, tumor promotion and carcinogenesis, and AIDS [6, 7]. Antioxidants are potent scavengers of free radicals and serve as inhibitors of neoplastic processes [8]. A large number of synthetic and natural antioxidants have been demonstrated to induce beneficial effects on human health and disease prevention. *In vitro* studies have shown significant antioxidant activity for specific dietary flavonoids (catechin, epicatechin, quercetin, and anthocyanins) and some of the major metabolites and conjugated derivatives that occur in the circulation after consumption of dietary flavonoids [9]. Because of the diverse chemical structures of flavonoids and their metabolites, they can have hydrophilic or relatively lipophilic properties and may interact with plasma proteins as well as the polar surface region of phospholipid bilayers in lipoproteins and cell membranes [10].

Because of the nature of these interactions, flavonoids may have the ability to protect against free radical attack in both aqueous and lipid environments, thus providing an effective antioxidant defense in biological systems. Oligomeric proanthocyanidins, naturally occurring antioxidants widely available in fruits, vegetables, nuts, seeds, flowers and barks, have been reported to possess a broad spectrum of biological, pharmacological and therapeutic activities against free radicals and oxidative stress. Proanthocyanidins present in grape seeds are known to exert anti-inflammatory, anti-arthritic and anti-allergic activities, and prevent skin aging. They also scavenge oxygen free radicals and inhibit UV radiation-induced peroxidation [11].

Grapes (*Vitis vinifera*) are one of the most widely consumed fruits in the world. Grape seeds are rich in dimmers, trimmers and other oligomers of flavan-3-ols (the major are catechin, epicatechin and epicatechin-3-O-gallate), named proanthocyanidins (PAs) [12]. There is a growing interest in the utilization of PAs for their dietary and pharmacological properties, especially positive effects on vascular injury [13], capillary protective action [14], free radical scavenging [15] and antimutagenic activity [16]. Oral administration of grape seed proanthocyanidins at a dose of 2 mg/kg three times daily for 6 days inhibited carrageenin- α or dextran-induced hind paw edema, stabilized the capillary wall and prevented the increase in capillary permeability caused by local cutaneous application of xylene [17]. The antioxidative activities of proanthocyanidins were found to be much stronger than vitamin C or vitamin E in aqueous systems [18-20].

The potential influence of grape seed extract on the bleomycin-induced fibrosis has not been previously reported. The aim of the present study was to examine the effects of orally administered grape seed extract in a rat model of lung injury produced by

endotracheal bleomycin by comparing it with that of α -tocopherol (vitamin E).

2. Materials and methods

2.1. Plant Material

The ripen grapes, originally from Hamadan province west of Iran, were purchased from local market in Ahwaz, south west of Iran, in July 2005. The plant was identified as *Vitis vinifera* (domestic name of big red grape) in the Department of Pharmacognosy, Faculty of Pharmacy, Jundishahpour University of Medical Sciences, Ahwaz, Iran. A sample of plant is kept in the Faculty herbarium with the number: A-06283001-P. The seeds were separated from the pulp and dried in shade. The dried seeds were powdered by a grinder. The powdered grape seed (200 g) was macerated in 70% ethanol for 72 h in laboratory temperature (25-30 °C). The filtrate extract was evaporated under vacuum below 45 °C in a vacuum drier to give a final yield of 24 g (12%, w/w) [21]. All chemical reagents used were of analytical grade.

2.2. Animals

Male NMRI rats (6-8 weeks old) weighting 250-300 g were obtained from Razi Vaccine Institute, Tehran, Iran. They were housed in standard stainless-steel cages at a 12 h cycle of light and dark. Room temperature was kept at 24 ± 2 °C and humidity maintained at 50%. Rats were allowed to become acclimatized to standard laboratory condition for at least 5 days and standard food and water was provided ad libitum.

2.3. Experimental procedure

Rats were anesthetized with intraperitoneal injection of ketamine hydrochloride (50 mg/kg). To produce pulmonary fibrosis, animals received a single dose of bleomycin (7.5 IU/kg dissolved in normal saline) endotracheally by the transoral route. Control animals were subjected to the same protocol but received the same volume of intratracheal

saline instead of bleomycin.

2.4. Experimental groups

The animals were randomly divided into six experimental groups which each group containing 7 rats. Group 1, negative control, received normal saline; group 2, positive control, received a single dose of bleomycin; group 3, received a single dose of bleomycin + vitamin E (20 IU/kg dissolved in sunflower oil); and groups 4 to 6 received a single dose of bleomycin + grape seed extract 100 mg/kg, 200 mg/kg and 400 mg/kg, respectively. Vitamin E or grape seed extract (0.3 ml) was administered orally 1 h after bleomycin on a daily basis (at 9:00 AM) for 14 days. Fourteen days after endotracheal bleomycin or saline, the animals were killed by ether and the lung samples were taken for biochemical and histopathological examinations.

2.5. Biochemical studies

Lung hydroxyproline content was measured as outlined by Woessner [22]. One hundred mg of the left lung tissue samples were homogenized and then hydrolyzed in 10 ml of 6N HCl for 18 h at 120 °C. The hydrolysate was then neutralized with 2.5 M NaOH. Aliquots (2 ml) were analyzed for hydroxyproline content after the addition of 1 ml of chloramine-T, 1 ml of perchloric acid, and 1 ml of dimethylaminobenzaldehyde. The absorbance of samples was measured at 550 nm in a spectrophotometer (Shimadzu UV-1650CT). Total collagen content was determined by multiplying the hydroxyproline content by a factor of 8 (based on hydroxyproline representing approximately 12.5% of the amino-acid composition of collagen, in most mammalian tissues) [22]. Results are expressed as μ g of hydroxyproline and collagen per gram lung tissue.

2.6. Histopathological studies

Lungs were first perfused by its main bronchus with a fixative solution (10%

neutral-buffered formalin) at a pressure of 25 cm H₂O, immersed in the fixative for 12-24 h, and blocks were taken. Tissue blocks were placed in formalin, dehydrated in a graded series of ethanol, embedded in paraffin, cut into 4 µm-thick serial sections, and stained with haematoxylin-eosin to identify inflammatory cells, connective tissue and collagen deposition.

2.7. Statistical analysis

Data are expressed as mean±SEM. Statistical analysis was carried out by analysis of variance (ANOVA) followed by appropriate post hoc tests including Tukey-test and Tamhane. $p < 0.05$ was considered significant.

3. Results

3.1. Lung weights

The lung weights of rats received bleomycin showed a significant increase after 14 days of treatment, compared to groups received normal saline, vitamin E or grape seed extract (Figure 1).

3.2. Hydroxyproline and collagen content of lung tissues

Hydroxyproline levels, a marker of collagen deposition, were increased at 14

days after bleomycin exposure, and treatment with grape seed extracts significantly reduced the hydroxyproline content in bleomycin-treated rats, although levels remained higher than those found in animals not exposed to bleomycin (Figure 2).

3.3. Histopathology

Haematoxylin-eosin stained lung sections were examined by light microscopy to determine whether bleomycin-induced pulmonary fibrosis was decreased by treatment with grape seed extracts. Lungs of rats in group 1 were histologically normal and showed no sign of acute inflammation or fibrosis (Figure 3). Lungs from rats in group 2 (positive group received bleomycin) at 14-days post-exposure showed marked peribronchiolar and interstitial infiltration with inflammatory cells (predominantly mononuclear cells including macrophages and lymphocytes with fewer numbers of neutrophils and scattered eosinophils), extensive thickening of interalveolar septa, interstitial oedema, increase in interstitial cells with a fibroblastic appearance and in interstitial collagen deposition were seen. Focal cuboidal metaplasia of alveolar lining cells was also detected. The pattern of distribution of lesions was multifocal (i.e.

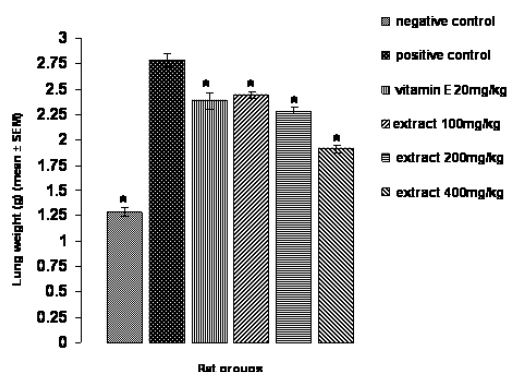


Figure 1. Lung weights of rats at the end of treatment period (14 days). *Significantly different from bleomycin treatment group ($p < 0.05$).

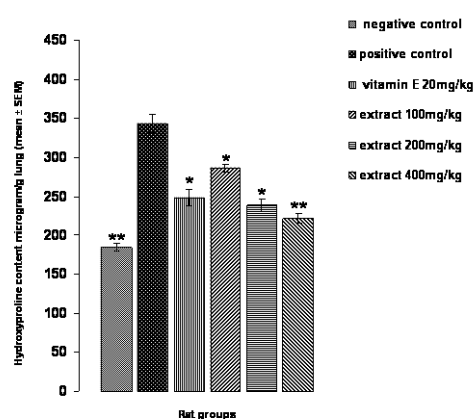


Figure 2. The hydroxyproline content of rat lungs treated with bleomycin alone; bleomycin + grape seed extract; or bleomycin + vitamin E. Values significantly different from bleomycin-treated group are indicated as * ($p < 0.05$) or ** ($p < 0.001$).

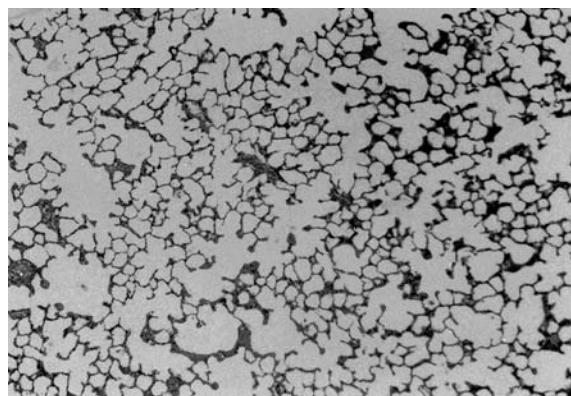


Figure 3. Photomicrograph of normal lung section. No septal thickening or inflammatory cells in alveolar spaces are seen (H&E $\times 50$).

patchy areas of pulmonary fibrosis) in most cases, commonly involving the pleura. In contrast, grape seed extracts-treated animals (groups 4-6) showed a less severe pattern of pulmonary lesion, dependent to dose, consisting of multifocal areas of moderate inflammation and slight fibrosis (Figures 4-9). Similar improvement was observed in sections from vitamin E treated group (Figure 10).

4. Discussion

Pulmonary fibrosis is a chronic inflammatory interstitial lung disease with a potentially fatal prognosis and a poor response to available medical therapy. Many studies have been done to ameliorate the life

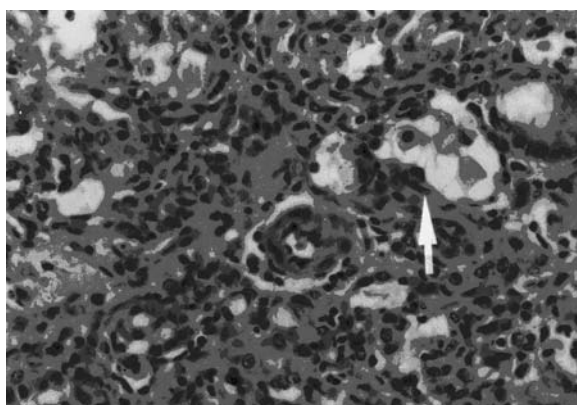


Figure 5. Photomicrograph of lung section 14 days after single bleomycin administration (positive control). Inflammatory cell infiltration e.g. macrophages, monocytes and association with fibroblast (arrow head) proliferation is evident (H&E $\times 530$).

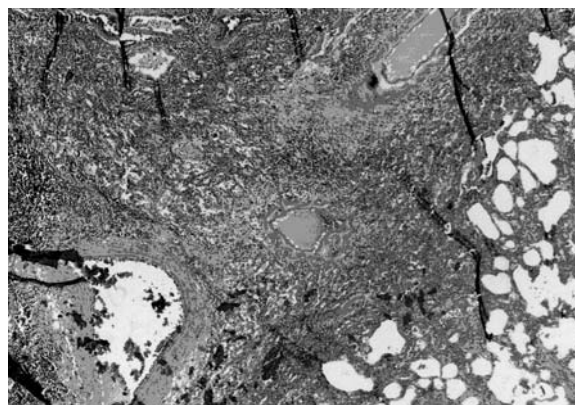


Figure 4. Photomicrograph of lung section 14 days after single bleomycin administration. Acute reduction in alveolar spaces associated with inflammatory cell infiltration has resulted in extensive fibrosis (H&E $\times 50$).

threatening effect of lung fibrosis; however, we might be at the beginning of the way to cope with this disease. One of the clinically relevant causative agents of pulmonary fibrosis is the antineoplastic agent, bleomycin, which is widely used in animal models to cause oxidant-induced inflammatory and fibrotic lesions in lungs [8]. This model of pulmonary fibrosis is useful to assess potential therapeutic agents including antioxidants and other drugs. Studies showed that when dietary flavonoids from food sources are absorbed from the gut, the circulating species are almost entirely conjugated, and that many of these conjugated metabolites have antioxidant properties *in vitro* [7, 9]. Beneficial effects of grape seed extract have been studied on

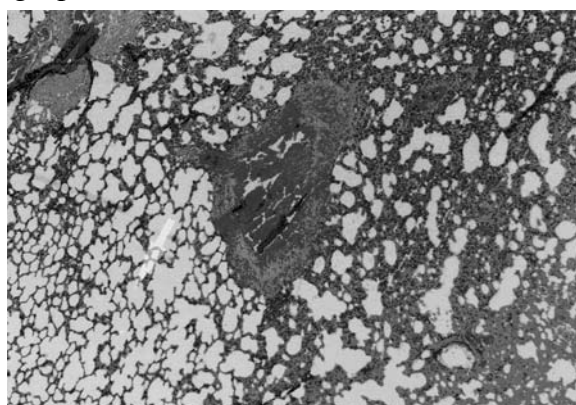


Figure 6. Photomicrograph of lung section 14 days after single bleomycin + oral grape seed extract (100 mg/kg/day) administration showing less alveolar thickening and reduced fibrosis (H&E $\times 50$).

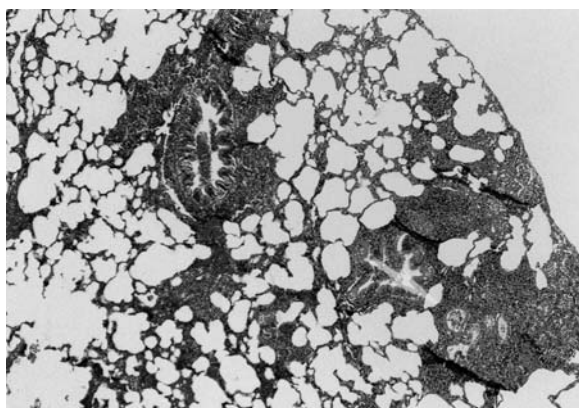


Figure 7. Photomicrograph of lung section 14 days after single bleomycin + oral grape seed extract (200 mg/kg/day) administration showing reduced alveolar thickening and scattered fibrosis (H&E x53).

variety of diseases [15, 17]. But preventing effect of grape seed extract on lung fibrosis has not been reported yet. Therefore, this study might be the first report on the effect of grape seed extract on lung fibrosis.

Hydroxyproline content of lung tissue is a good indicator for the development of fibrosis as is it associated with the collagen deposition in tissue. Therefore, one of the major objects of this study was to verify the hydroxyproline and subsequently the collagen content of lung in rats. Our study demonstrated the efficacy of grape seed extract to reduce the fibrogenesis induced by bleomycin. Such an

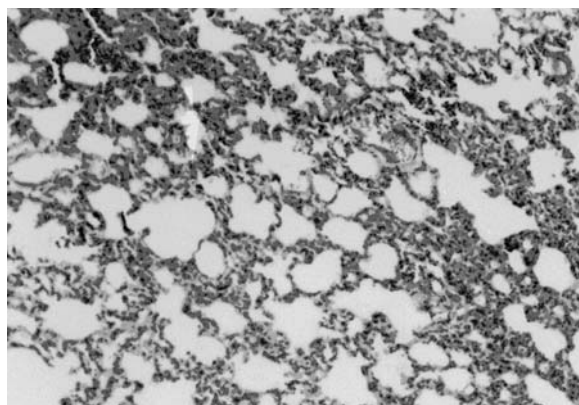


Figure 9. Photomicrograph of lung section 14 days after single bleomycin + oral grape seed extracts (400 mg/kg/day) administration. More alveolar spaces are opens and pronounced reduction in fibrosis is seen. Interstitial pneumonia is mainly evident (H&E x53).

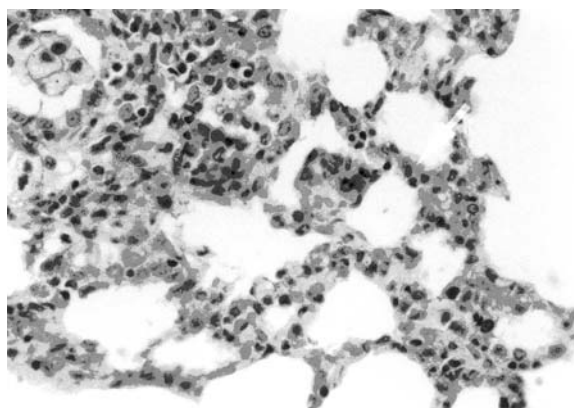


Figure 8. Photomicrograph of lung section 14 days after single bleomycin + oral grape seed extract (200 mg/kg/day) administration showing less alveolar thickening and reduced numbers of inflammatory cell proliferation (H&E x532).

effect was dose dependent as was shown by histopathology and hydroxyproline analysis. This study may not be able to elucidate the mechanisms involved in the effect of grape seed extract on pulmonary fibrosis. However, the free radical scavenging effect of antioxidants and the modulating effect of proanthocyanidin [23] and other constituents of grape seed extract may be responsible for such effect. Recent researches verify the significant roles of cytokines in pulmonary fibrosis [24]. Inhibition of the formation of inflammatory cytokines by proanthocyanidin, present in grape seed, has also been

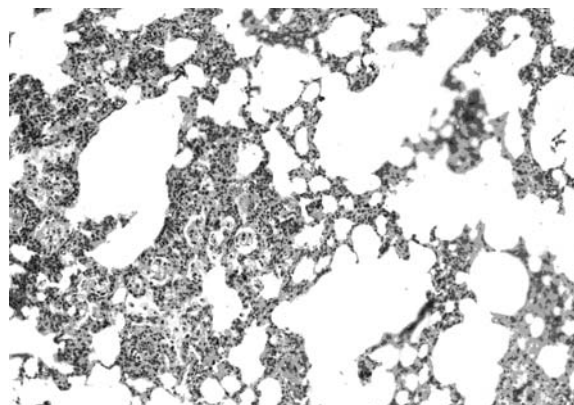


Figure 10. Photomicrograph of lung section 14 days after single bleomycin + oral vit E (20IU/kg/day) administration. Inflammatory cell infiltration associated with focal fibrosis is seen (H&E x53).

reported in croton oil induced ear swelling in mice, and in carrageenan-induced hind paws edema of rat [25]. One possibility of positive effect of grape seed extract in pulmonary fibrosis can be attributed to the inhibition of release of cytokines e.g. transforming growth factor beta (TGF β) in fibrotic lungs [26]. This hypothesis needs further studies to be proved. Nevertheless the use of grape seed extract has its own advantage of being natural product with high safety margin due to edible nature of grape and its seeds. But in order to elucidate the exact mechanisms including molecular mechanism of grape seed extract in lung fibrosis more studied need to be done.

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