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Control the bleomycin-induced pulmonary fibrosis by a combination of hypericum extract and niacin

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ABSTRACT

Pulmonary fibrosis is a fatal disease, and is considered as one of the most important side effects of chemotherapy drugs such as bleomycin. The antioxidant properties and anti-inflammatory activities of Hypericum have been confirmed; on the other hand, the inhibition of poly (ADP-ribose) polymerase enzyme affected the levels of nicotinamide adenine dinucleotide (NAD) in cells. In the present study, the effects of hypericum perforatum extract in combination with niacin were assessed on bleomycin-induced pulmonary fibrosis. In this study, male rats weighing 160-190 g were used. The animals were randomly divided into the following five groups of six: The control group (NS) , which receives normal saline only; the bleomycin group (BLM) , which was administrated with an endotracheal single dose of bleomycin sulfate solution after anesthetizing the animals. Hypericum perforatum (HP) group, Niacin group (100 mg/kg), and the combination group (HP + NC). In addition to receiving endotracheal bleomycin, the studied drug was administered intraperitoneally for 21 days. At the end of the study, hydroxy-proline and malondialdehyde were measured, and specific pathology tests were performed in lung tissues to determine the changes that occur in in various groups. The results showed that the lung index (lung weight/body weight), hydroxy-proline content (μg of hydroxyproline/lung), and malondialdehyde in the control group were 0.662±0.03 μg, 1156.8±41.5 μg ,and 753.7±40.2 μg respectively, while these values in the BLM group were 1.56± 0.24 μg, 4073.4±468.9 μg, and 1643.6 ± 129.9 μg, respectively. Treatment with the combination of hypericum extract and niacin significantly reduced these factors as compared to the bleomycin group ($P < 0.05$) .Our results represented a potential protection of hypericum extract and niacin on lung fibrosis induced by bleomycin. This protective effect showed a significant increase when compared to the use of the plant extract or niacin alone.

Keywords: Bleomycin, Pulmonary fibrosis, Hypericum, Niacin

INTRODUCTION

Pulmonary fibrosis is an interstitial lung chronic disease that is caused by lung parenchyma damage by factors such as generating inflammation and fibrosis. This resistance to treatment disease is associated with high mortality rates [1]. The average survival rate for this disease accounts for about 2-3 years [2]. The pathogenesis of pulmonary fibrosis are not clearly defined [3,4];however, several factors such as reactive oxygen species (ROS), growth factors, inflammatory cells such as lymphocytes and neutrophils, cytokines, and chemokines play a major role directly or indirectly in the fibrotic process. Some of the known factors that are involved in the incidence of this disease can be mentioned as the following: Viral infections caused by some herpes viruses, damage resulting from some inorganic

compounds such as asbestos and silica, and finally, the side effects of some chemical drugs such as bleomycin and methotrexate [5]. Bleomycin is a strong antineoplastic antibiotic, which has been effective in the treatment of squamous cell carcinoma of head and neck, testicular carcinoma, some smooth tissues sarcoma, and Hodgkin's disease [6]. Due to low blood side effects, bleomycin is valuable in chemotherapy [7,8]. Bleomycin-induced pulmonary fibrosis mechanisms are not completely identified, but there are some hypotheses.

Drugs can chelate the iron and copper elements and then bind to activated oxygen molecules and create complexes, which will attach to the DNA molecule. In the next step by transferring electrons from Iron II to the oxygen molecules generates free radicals of hydroxyl and superoxide. Consequently, inflammation, accumulation of inflammatory cells, and finally, collagen deposition had occurred in the walls of the air sacs [7,9]. The immune system appears to be the most important factor in bleomycin-induced pulmonary toxicity [10]. Cytokines are hormone-like or pseudo growth factor proteins that induce cell specific activities mediated with specific cell receptors.

The most important pro-inflammatory cytokines involved in fibrosis process include interleukin-1, interleukin-6, interleukin -13, TNF-α, and TGF-β [11,12]. Prostaglandins, leukotrienes, and thromboxane are major inflammatory factors synthesized in the course of arachidonic acid that can be involved in the development of pulmonary fibrosis [13]. Hypericum, (Alaf Chai or Raie Flower) with the scientific name of Hypericum perforatum, is a genus of herbaceous, flowering, perennial plant, 10-110 cm in height, and without trichome. Its flowering branches and flowers are used as medicinal parts [14]. More than ten important compounds have been found in Hypericum, which generally contain flavonoids, phloroglucinols, and naftodiatrons [15].The major flavonoids in Hypericum are quercetin and luteolin.

In the past researches, the amount of flavonoids in the plant flowers, leaves, and stem were calculated 11.7% and 7.4%, respectively [16]. The plant has been mostly used in the treatment of depression and neurological disorders [17, 18]. Several studies have shown that the flavonoids and hyperforin in Hypericum are mostly responsible for anti-inflammatory and analgesic effects, anti-bacterial and anti-viral properties, changing in capillary permeability, anti-arrhythmic effects, coronary vasodilatation properties, antispasmodic effects,and changing the strength and speed of heart contraction [19]. Researchers have confirmed the strong anti-inflammatory and anti-pulmonary fibrotic properties of Hypericum extract [16, 20, 21].

The most complications of Hypericum are hypersensitivity to light and skin erythema [18]. The poly (adenosine diphosphate ribose -5) polymerase is the enzyme related to the cell nucleus, which is activated due to damages related to nucleic acids for their repair [1]. This polymerase uses NAD molecules as its substrate, and leads to a rapid decrease of f the cells NAD content. As a consequence, the glycolysis pathway interrupted and the levels of Adenosine triphosphate (ATP) of the cell would diminish [22]. As a result, the active calcium pump stops and the calcium accumulate inside the cell and lead to cell death [23]. According to previous studies, niacin or nicotinic acid can probably be effective in the treatment of bleomycin-induced pulmonary fibrosis by inhibiting the mentioned enzyme, as well as to the leading substance of NAD synthesis and proving ATP required by the cell [1, 24].

MATERIALS AND METHODS

Methodology and required materials

Bleomycin sulfate was purchased from the United Biotech Company, India. Ketamine ampoule was prepared from Davis Park factory and other compounds such as hydroxyproline, chloramine-T, para-Dimethylaminobenzaldehyde, and niacin with high purity were obtained from Merck Company, Germany.

Preparing the extract

The Hypericum plant was purchased from Gol Daroo herbal medicine company (Isfahan, Iran). The plant was harvested in June, and it was identified with the herbarium number of 11427 in Isfahan Center for Research of Agricultural Science and Natural Resources. The soaking method was used to prepare the extract [25, 26]. First, the Hypericum head branches were dried quickly in the shadow. Then, they were chopped into small pieces (2-3 cm), and soaked for 72 hours in 70% ethanol solution (water: 30; ethanol: 70). After three days, the extract was filtered. The 70% ethanol was poured to the remaining pulp and added to the first extract. The obtained extract was then passed through filter paper, and the filtrate was concentrated by rotary device. Then it was followed by putting the solution in the Dry Heat on 30-40° C, and lastly the dry extract was obtained [25, 27, 28].

Laboratory animal

Thirty 14-week old adult male Wistar rats weighing from about 160-190 g were used in the study (the Faculty of Pharmacy, Isfahan University of Medical Sciences). The animals were transferred to Professor Abu-Turab Research

Centre a week before the start of the experiment, and were randomly placed in standard cages (4 rats per cage). The animals were kept under controlled environmental conditions (alternate light and dark cycles, 12 h each, the temperature was maintained at 22±3°C) and were provided with food and water.

The animals were randomly divided into the following five groups of six: Control group (NS) that received normal saline only; bleomycin group (BLM), which were anesthetized with ketamine (50 mg/kg); an endotracheal which was administrated with a single dose of bleomycin sulfate solution (1u/100g of body weight) ; Hypericumperforatum (HP) group received only plant extract (200 mg/kg); Niacin group received niacin complement (100 mg/kg) ;lastly, the Combination group (HP + NC) was administrated by a Hypericum perforatum (200 mg/kg) with niacin (100mg/kg).

Preparing the pulmonary fibrosis model

Bleomycin sulfate was dissolved in sterile normal saline. After anesthetizing the animals with ketamine (50mg/kg), a tracheal tube was put in the animal's mouth through which the drug solution was instilled into the animal lung [29]. The animals in BLM group and the treatment groups were all endotracheally administrated with 0.3 ml of solution drug, containing1u/100g bleomycin, while the control animals received only 0.3 ml normal saline endotracheally. Then, all the rats were kept under supervision until complete recovery. In addition to bleomycin, the HP group received 0.5 ml of the plant extracts solution intraperitoneally (equivalent to 200 mg/kg) daily for about 16 days. The NC group received niacin solution intraperitoneally daily for 16 days. In addition, the HP+NC group received 0.5 ml of hypericum perforatum extract solution (equivalent to 200 mg/kg) and niacin solution (equivalent to 100 mg/kg) in two separate intraperitoneal injections every day. The bleomycin and the control groups were also daily intraperitoneally injected by 0.5mL of normal saline. In all groups, seven days before bleomycin administration, the examined drugs were injected intraperitoneally (pretreatment). The doses used in this study were determined based on previous studies that have proven the preventive and protective effect of niacin on bleomycin-induced pulmonary fibrosis [1] and the impact of the plant extract on inflammation and pain caused by formalin according to the previous studies that have examined the effects of similar plants extract on bleomycin-induced pulmonary fibrosis [30]. No toxic and threatening side effects were reported regarding the doses of extract used in the conducted studies. In contrast, the protective effects of this plant on drug poisoning caused by toxic drugs on liver and kidneys have been examined and documented [31].

Sampling

After measuring the animals' body weight, they were anesthetized by ketamine injection. Then, the animal's chest was cut and the lungs were removed carefully. The rats' lungs weight was measured. After washing the lungs with cold normal saline, the left lung was separated for histological studies and a part of it was placed in 10% formalin solution. For histopathological study, six slides were prepared from each group (one slide from each animal), and five fields per slide were examined. The rest of the lung tissues were kept in special container in the freezer at -70 degrees centigrade for biochemical tests.

Determining hydroxyproline content in the lung tissue

The Woessner method was used to determine the value of hydroxyproline, which reflects the lung collagen content. One-third of the lung tissue was homogenized with 10 ml of sterile water; then,1ml of the homogenized liquid of lung tissue was taken. After precipitation of its proteins with 10% TCA, the precipitates were hydrolyzed with hydrochloric acid 6 N for 16 hours at a temperature of 120°C. The samples were then neutralized by sodium hydroxide (NaOH) 2.5 normal(PH 6-7). The oxidation phase was started by adding 1ml of chloramine T reagent (1.41 g chloramine T in 20 ml of water, 30 ml of isopropyl alcohol and 50ml of citrate-acetate buffer) to 2ml of the neutralized sample. After 20 minutes, 1ml of Ehrlich reagent(20% P-dimethyl amino benzaldehyde solution in perchloric acid and isopropyl alcohol) was added and the samples were incubated for 20 minutes in a water bath with a temperature of 60 °C. After incubation, the tubes were placed in cool tap water for 5 minutes, followed by determination of the absorbance at 557 nm. By using the hydroxyproline standard, the hydroxyproline concentration in the samples was determined and the total hydroxyproline content of lung was calculated. The results were reported as hydroxyproline content in the entire lung (µg/lung) [1, 2, 32, 33].

Determination of lung tissue malondialdehydecontent (34)

Malondialdehyde (MDA) content of lung tissue was determined by using the thiobarbituric acid method as an indicator for the tissue lipid peroxidation rate. The proteins of 1ml of the homogenized sample (0.3g of lung tissue in 3ml normal saline) were precipitated by trichloroacetic acid (10% w/v). Then, the supernatant liquid was isolated to measure MDA; thus, 0.6ml of the solution was mixed with 1.2ml of thiobarbituric acid (0.67 w/v) (35). The samples were placed in a bath of boiling water for half an hour at 80°C. Then, after adding N- butanol to samples, absorption was immediately measured at 532nm by spectrophotometry, and the amount of MDA was reported in nmol per gram of lung tissue (nmol/g tissue) [34, 36].

Histological studies

For histological studies, the formalin-fixed samples were sectioned at 4µm pieces after dewatering in paraffin, and the slides were prepared. After staining them with Hematoxylin-Eosin and Masson's trichrome, the slides were examined by light microscopy [2, 32].

Statistical analysis

The SPSS version 16 was used for analyzing the data. All the results are expressed as mean values ± standard error mean. Variations between the groups were compared by using the ANOVA and Duncan test. A P-value of less than 0.05 was considered to be statistically significant and the excel software was used to draw the charts, curves, and graphs of the results.

RESULTS

The study results showed that the level of malondialdehyde of lung tissue in NS and BLM groups was 753.7 ± 40.2 and 1687.5 ± 218nanomoles per gram, respectively($p < 0.05$).The values of MDA of lung tissue in HP, NC ,and HP + NC groups were 904.5 ± 78.4,1130.6 ± 92.4and 655.2 ± 42.1 nanomoles per gram, respectively (Figure 1). The value of OH-Prolin of lung tissue in NS and BLM groups were1156.83 ± 41.5 and 4.73.4 ± 468.9 micrograms ,respectively ($p < 0.05$).The OH-Prolin content in the lung tissue in the HP, NC, and HP + NC groups were 2781 ± 188.3,2163.8 ± 155.13, and 1638.6 ± 161respectively (Figure 2). The lung index in groups NS and BLM was as 0.662 ± 0.03 and 1.56 ± 0.24 respectively ($p <0.05$). The rate of lung index in HP, NC, and HP + NC groups were as 0.922 ± 0.11, 1.16 ± 0.13 and 0.809 ± 0.09 respectively (Figure 3).

Results of histological studies

Bleomycincreates many histological changes in the lung tissue, including thickening of the alveolar wall, accumulation of fibroblasts, and connective tissue in the alveolar space filling of these spaces. Microscopic observations of lung tissue in different groups of animals based on H & E staining are shown below. According to Figure 4, microscopic observations of lung tissue in various animal groups by H&E staining were demonstrated in the groups that received normal saline, and the lung that have a normal view and alveoli and interstitial tissue are normal with no observable destruction. Also, accumulation of inflammatory cells and collagen fibers or fibrosis was not observed (Figure 4A). In bleomycin groups, the following changes were observed: accumulation of fibroblasts and connective tissue in the alveolar space, developed destruction of the alveoli, accumulation of collagen fibers in some areas of the lung. A visible hemosiderin pigment deposition in the picture is a sign of previous bleeding (Figure 4B). In the group receiving bleomycin and niacin, inflammatory and fibrotic lesions have significantly decreased as compared with the bleomycin group; however, the thick walls of the alveoli and inflammatory cells were seen in many areas of tissue (Figure 4C).In the group receiving bleomycin and Hypericum perforatum extract, in comparison with the previous group, a reduction in lung injury by inhibiting the infiltration of inflammatory cells and reduced thickness of the walls between alveoli were observed (Figure 4D).In the groups receiving bleomycin and Hypericum perforatum extract associated with niacin, most areas of the tissue have almost normal structure, and the lung tissue damage has been greatly reduced (Figure 4E).

DISCUSSION

Fatal pulmonary fibrosis disease is one of the side effects of anti-cancer drug bleomycin, which has many applications in chemotherapy [6, 37]. In the reaction process with DNA, the presence of molecular oxygen in the air, bleomycin increases the ROS production. These active agents attack cell membranes in the lungs and cause the production of lipid peroxide and other dangerous active radicals. Due to the accumulation of bleomycinin the lungs as well as the availability of molecular oxygen in the site, this process progress as chain reactions[32].

Through extensive immunological reactions, bleomycin causes damage in the alveolar cells, increases the accumulation of inflammatory cells, collagen tissue and ECM in the alveolar area, stimulates the proliferation of fibroblast cells in the alveolar interstitial area, and finally develops fibrosis. It is also said that bleomycin alters the metabolism of eicosanoids. These changes, especially on the PGs and thromboxane, are more likely to play an important role in fibrosis formation [2, 5]. It is also believed that bleomycin increases the production of TGF-B protein, which is a growth factor with a key role in the process of fibroblast proliferation and developing pulmonary fibrosis [38]. Despite much research, the causes and pathogenesis of pulmonary fibrosis are still unclear. Much research has been done so far on inhibiting this type of pulmonary fibrosis, and many compounds have been introduced for the purpose of preventing and treating each act with a certain mechanism. In this study, the effects of Hypericumperforatum extract in combination with niacin were investigated 16 days after endotracheal injection of bleomycin according to the measurement of lung tissue hydroxyproline content as an indicator of collagen tissue, lung tissue MDA as an indicator of oxidative stress [34, 39], and pulmonary weight index (lung weight to body weight in percent) as well as histological changes. Hypericum is a medicinal plant that is mostly used in cases of

depression. Having flavonoids like quercetin, rutin, and anthocyanoside can act as an antioxidant, anti-inflammatory, and analgesic substances[40]. Research shows that through lowering cytokines of TNF-α, IL-6 and IL-1 [41],as well as influencing the synthesis path of arachidonic acid, and inhibiting the production of eicosanoids [42], the flavonoids can reduce the inflammation. Flavonoids act as antioxidants. In addition, by increasing the capacity of antioxidant enzymes of glutathione, glutathione reductase, glutathione peroxidase, and catalase can protect cells against reduced glutathione discharge[41]. With chelating iron as a factor in the development of free radicals, they can exert their beneficial effects [8]. Also, by reducing the number of immobile leukocytes and theinhibition of neutrophils degranulation, flavonoids prevent the stabilization and adhesion of leukocytes to the endothelial wall and the formation of oxygen free radicals, inflammatory mediators, and further complement activation. Flavonoids also reduce inflammation by limiting the activity of NF-Kb [41, 43].

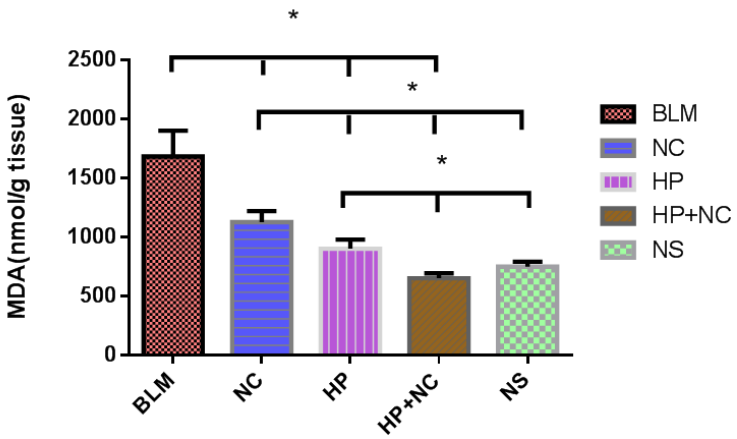

Figure 1: levels of lung tissue malondialdehyde in the study groups in terms of nanomoles per gram of lung tissue
*Data are expressed as the mean nanomoles per gram of malondialdehyde of lung tissue ±SD. *P < 0.05.*

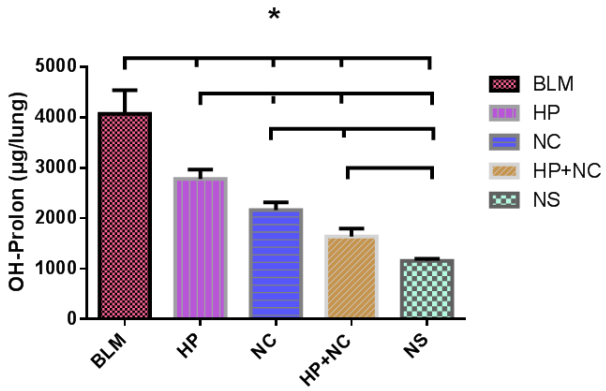

Figure 2: The effects of different examined groups on the rate of lung tissue hydroxyproline in the study groups in terms of micromoles per gram of total lung tissue
**: p <0.05*

Niacin, or nicotinic acid, is a B-complex vitamin that has been used in the past to treat pellagra. Studies show that niacin can provide the ATP required by the cell to repair and reconstruct DNA and cellular components through provision of cell NAD, which bleomycin has discharged it, and thereby, it will increase the lesions repair, and therefore, prevents the development and progression of fibrosis [22]. Influencing the NF-Kb pathway and the body immune system, Niacin can also reduce inflammation, resulting in decreased bleomycin-induced pulmonary fibrosis [24].

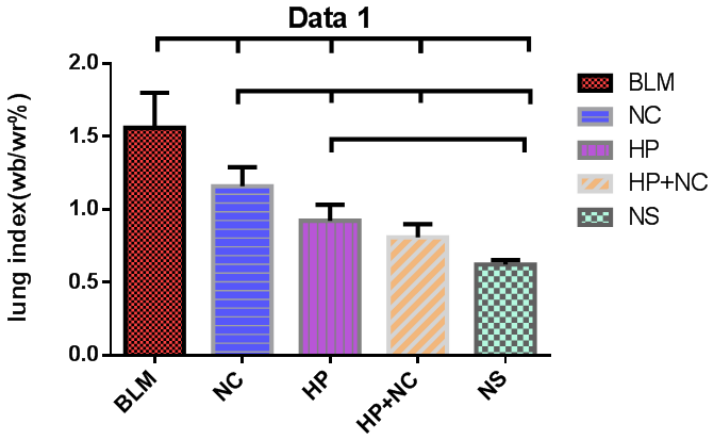

Figure 3: Effects of different examined groups on the lung index (gram body weight in percent / g lung weight) in the study groups. *: ($p < 0.05$)

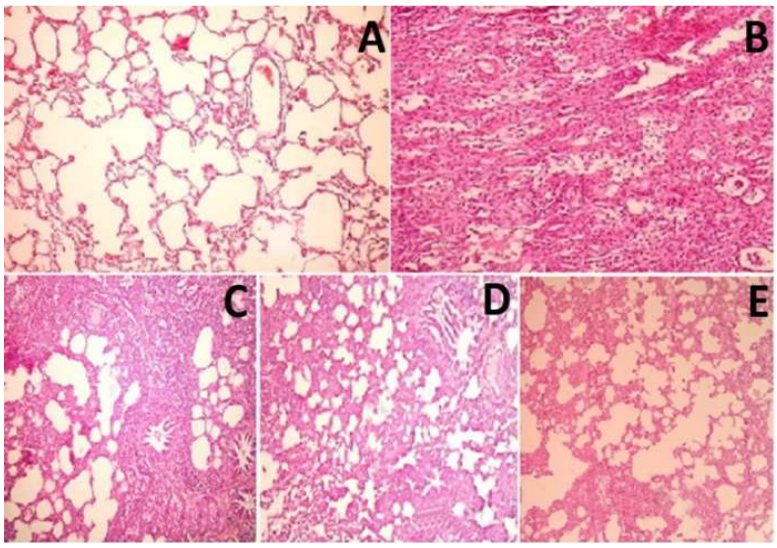

Figure 4:Hematoxylin and eosin-stained lung sections. Lung sections tissue from normal saline (NS) rats (A), rats receiving bleomycin only (B), rats receiving bleomycin and niacin together (C), rats receiving bleomycin and Hypericumperforatum extract together (D) andrats receiving bleomycin and Hypericumperforatum extract associated with niacin Original magnification: x40

Studies conducted in the past indicate that niacin alone can effectively prevent the development of bleomycin-induced pulmonary fibrosis. In a study conducted by Gray and Javadi in 1990, administrating niacin at doses of 500 mg/kg on a hamster animal model, they could control pulmonary fibrosis to an acceptable level so that the rate of lung tissue hydroxyproline in the bleomycin group increased as 258% compared to the control group, while this rate reduced to 230% in niacin treatment group [1].According to previous studies, flavonoid luteolin can improve the inflammation effects and skin redness caused by niacin intake by inhibiting prostaglandin D[44].Niacin can also increase the absorption of flavonoids in the Hypericum[42, 45].

In conclusion, one can say that antioxidant agents such as flavonoid containing compounds can increase the antioxidant capacity of the body as pre-treatment. Thereby, in the early stages of fibrosis, they can reduce initial damage and pulmonary fibrosis by inhibiting free radicals. In continuation of the fibrosis process, the anti-inflammatory properties of these compounds can inhibit fibrosis through primary reduction of inflammation. As a result, one can say that the combination of niacin and hypericum extract can reduce fibrosis by inhibiting free radicals and inhibiting inflammatory factors, and providing NAD required for the repair of damaged cells.

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