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Original Article

A STUDY ON THE PROTECTIVE EFFICACY OF *BRASSICA RAPA CHINENSIS* AGAINST BLEOMYCIN INDUCED PULMONARY FIBROSIS

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ABSTRACT

Objective: Bleomycin (BLM) is a polypeptide antitumor antibiotic agent isolated from a strain of *Streptomyces verticillus*. Inspite of the fact that BLM is widely used in the treatment of tumors, it has been reported to cause pulmonary fibrosis. The work is aimed to evaluate the effect of the aqueous extract of *Brassica rapa chinensis* against BLM induced pulmonary fibrosis.

Methods: Pulmonary fibrosis was experimentally induced in Sprague Dawley rats by s.c injection of BLM. Aqueous extract of *Brassica rapa chinensis* (250, 500 mg/kg, p.o) was administered to the rats orally and the effects of the administration were assessed.

Results: Aqueous extract of *Brassica rapa chinensis* (250, 500 mg/kg, p.o) showed significant protective effect against BLM induced pulmonary fibrosis in rats by normalizing the levels of glycoproteins (hexose, hexosamine and sialic acid) and improving the activity of Catalase (CAT) and Superoxide dismutase (SOD). The extract also improved pulmonary glutathione (GSH) content and depleted the lipid peroxidation levels in a dose dependent manner. Vitamin C was used as the standard drug. The histopathological analysis also reveal the reversal of the lung architecture to near normal upon administration of plant extract.

Conclusion: The results suggest that the extract at both the doses was able to significantly ameliorate the effects of BLM.

Keywords: Bleomycin, *Brassica rapa chinensis*, Bok choy, Pulmonary fibrosis.

INTRODUCTION

Pulmonary fibrosis is the end stage of a heterogeneous group of disorders of known and unknown etiology. Despite the wide variety of insults associated with this condition such as bacterial infection, inhalation of organic and inorganic dusts, radiation, drugs and trauma the mechanisms involved appear largely the same[1]. It is assumed that, in response to injury, inflammatory cells enter the lung and, together with resident lung cells, release mediators that stimulate fibroblast proliferation and collagen deposition within the lung interstitium.

Pulmonary fibrosis is a chronic inflammatory interstitial lung disease of potential fatal prognosis and poor response to available medical therapy. It has been hypothesized that activated inflammatory cells which accumulate in the lower airways, release harmful amounts of reactive oxygen species (ROS) that result in parenchymal injury, and interstitial and alveolar fibrosis[2].

Many xenobiotics that stimulate the over production of ROS, such as paraquat[3], butylated hydroxytoluene[4] and bleomycin[5] are capable of producing lung fibrosis.

Bleomycin (BLM) has been successfully used to treat a variety of tumors including squamous cell carcinoma of the head, neck, lung, cervix and oesophagus, as well as germ cell tumors, Hodgkin's and non-Hodgkin's lymphomas[6]. Moreover, BLM is used in combination chemotherapy regimens because of its broad activity and low myelotoxicity[7].

BLM has a selectively toxic effect on cells in mitosis and G2 phases of the cell cycle and generally more effective against actively dividing cells rather than resting ones[8]. The use of BLM is sometimes featured by the occurrence of severe side effects. After intravenous infusion, high concentrations of the drug are detected in the skin and lungs that become major sites of its toxicity. Resistance to BLM in normal tissues can be correlated with the presence of a bleomycin hydrolase enzyme. The low concentration of this enzyme in the skin and lung may explain the unique sensitivity of these tissues to BLM toxicity. It was recorded that the major limitation of BLM therapy is the potential for developing of pulmonary toxicity which most commonly takes the form of life-threatening interstitial pneumonitis and fibrosis. Pneumonitis can occur in up to 46% of patients treated with BLM-containing chemotherapy[9].

BLM-induced lung fibrosis in animals is a popular model for the study of human lung fibrosis[10]. Although the exact mechanisms by which BLM causes pulmonary fibrosis remain unclear, it is generally believed that ROS generated by BLM causes direct injury to the lung epithelial cells[11,10]. In experiments, BLM was shown to induce diffuse alveolar damage and pulmonary fibrosis in mice[12,13], hamsters[14] and rats[15,16].

Recently, increasing interests have been given to ROS generation in lung fibrosis [17,18]. ROS, such as superoxide anions, hydrogen peroxides, and hydroxyl radicals have been demonstrated to be an important mediator of BLM-induced lung fibrosis [19,20]. Excessive production of ROS is known to induce tissue damage or cell death, which could lead to several physiological and pathological processes.

Phytochemicals derived from plants are excellent antioxidants. Antioxidants appear to act against diseases by raising the levels of endogenous defense, by up-regulating gene expressions of the antioxidant enzymes [21,22].

The phytonutrients found in Bok choy are powerful antioxidants that are capable of strengthening your immune system. Intake of this vegetable could reduce the risk of osteoporosis.

The present study was undertaken to evaluate the pulmonary fibrosis potential of the aqueous extract of Bok choy, *Brassica rapa chinensis* Linn against BLM-induced lung injury.

MATERIALS AND METHODS

Preparation of the sample

Bok choy, *Brassica rapa chinensis* was obtained from local department store, Coimbatore, Tamilnadu, India. The leaves were cleaned, shade dried and powdered. 10g of the powder was extracted with 100 ml of water at 100^{0}C for 4 hours, centrifuged at 5000 rpm for 15 minutes and filtered through Whatman No.1 filter paper. The residue was extracted twice with 100 ml portions of water, as described above. The extracts were combined and vacuum evaporated. The extract obtained after vacuum evaporation was freeze dried and stored at 4^{0}C until further use.

Drugs and chemicals

Bleomycin was purchased from Cipla Ltd, Mumbai and Vitamin C was obtained from Himedia, Bangalore, India. All other chemicals used in this study were obtained commercially and were of analytical grade.

Experimental Design

Male Sprague Dawley rats weighing approximately 180-200g obtained from Small Animal Breeding Station, Thrissur, Kerala, were used for the study. The animals were maintained under standard conditions of humidity, temperature (25 ± 2ºC) and light (12 h light/dark). They were acclimatized to animal house conditions and were fed on a commercial pelleted rat chow (AVM Cattle Feeds, Coimbatore, and Tamil Nadu) and water *ad libitum.* Experimental animals were handled according to the university and institutional legislation, regulated by the Committee for the purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India. The animals were divided into 5 groups of five animals each. Pulmonary fibrosis was induced with subcutaneously (s.c) dose of BLM[23]

Group I : Control

Group II: Treated with BLM (15 mg/kg body weight (b.wt), subcutaneously.), three times a week for a total period of 4 weeks.

Group III: Treated with 250 mg/kg b.wt / day of aqueous extract of *Brassica rapa chinensis* (BRCAE) orally, to the animals for 7 days prior and during BLM induction.

Group IV : Treated with 500 mg/kg b.wt / day of aqueous extract of *Brassica rapa chinensis* (BRCAE) orally, to the animals for 7 days prior and during BLM induction.

Group V: Treated with 250 mg/kg b.wt / day of vitamin C orally, to the animals for 7 days prior and during BLM induction.

Biochemical Analysis

At the end of the last injection, the animals were subjected to fasting for a period of 12 hours. At the end of 12 hours fasting the animals were sacrificed, blood was collected and the lung were excised and washed in saline.10% homogenate of the lung tissues was prepared with 0.1 M Tri-HCl buffer, pH 7.4. Plasma was prepared from whole blood. The homogenates were centrifuged at 3000 rpm for 15 min at 4ºC for cytosolic separation.

The levels of Hexose and Sialic acid by the method of Niebes[24] and Hexosamine was determined by Wagner[25].

The enzymatic activity of pulmonary superoxide dismutase (SOD) was assessed according to the method of Das et al[26] and Catalase (CAT) by the method of Sinha[27], Glutathione (GSH) content of pulmonary tissues were assessed using Ellman's reagent according to the method described by Ellman[28]. Protein levels were determined as described by Lowry[29].

Rat lung homogenate lipid peroxide (LPO) levels were determined by measuring MDA content according to the method of Niehus and Samuelsson [30].

Histopathological Examination

The pulmonary tissue of each animal were dissected out and then fixed in buffered formalin for 12 hours and processed for histopathological examination. Four µm-thick paraffin sections were stained with hematoxylin and eosin for light microscope examination using conventional protocol.

Statistical analysis

The data are expressed as mean ± S.D. Statistical comparison was done at significance level, $p<0.05$ using SPSS package version 10.0. One way ANOVA followed by post hoc analysis of LSD was performed.

RESULTS

Table 1 represents the levels of hexose, hexosamine and sialic acid in the plasma of the experimental animals. There was observed a significant ($p<0.05$) raise in the levels in plasma of the BLM intoxicated rats. The treatment with BRCAE to the animals of group III and IV at a dose of 250 mg/kg and 500 mg/kg b.wt, respectively, resulted in a significant ($p<0.05$) decrease in the activity of the glycoproteins levels in a dose dependent manner. Vitamin C administration to the group V animals, also resulted in a marked reduction ($p<0.05$) in the levels of hexose, hexosamine and sialic acid.

Table 1: Effect of aqueous extract of *Brassica rapa chinensis* on the levels of Hexose, Hexosamine and Sialic acid in the plasma of experimental animals

Groups	Hexose (mg/dl)	Hexosamine (mg/dl)	Sialic acid (mg/dl)
Control	92.78 ± 5.29^b	25.46 ± 1.17^b	30.10 ± 1.15^b
Bleomycin (15 mg/kg b.wt)	186.07 ± 9.36^a	54.72 ± 3.09^a	63.79 ± 4.06^a
BRCAE (250 mg/kg b.wt) + Bleomycin	129.36 ± 8.62^b	38.06 ± 2.11^b	49.45 ± 2.87^b
BRCAE (500 mg/kg b.wt) + Bleomycin	101.09 ± 7.12^b	26.39 ± 1.03^b	29.73 ± 1.32^b
Vitamin C (250 mg/kg b.wt) + Bleomycin	99.88 ± 6.71^b	29.49 ± 2.11^b	33.70 ± 3.01^b

Group I- Control Group II- Bleomycin (15 mg /kg b.wt) Group III- BRCAE (250 mg/kg b.wt) + Bleomycin, Group IV- BRCAE (500 mg/kg b.wt) + Bleomycin, Group V- Vitamin C (250 mg/kg b.wt) + Bleomycin Values are expressed as mean ± SD for six animals. Group comparison and statistical significance at $p<0.05$: ^{a}: Group I vs. II, III, IV, V. $^{b:}$ Group II vs. I, III, IV, V.

Table 2:Effects of aqueous extract of *Brassica rapa chinensis* on the activity of SOD, CAT and the levels of MDA and GSH in lung of experimental animals

Groups	SOD (U/mg protein)	CAT (U/mg protein)	GSH (µg/mg protein)	MDA (nmoles /min/mg protein)
Control	7.88 ± 0.36^b	15.5 ± 0.76^b	5.33 ± 0.29^b	1.75± 0.09^b
Bleomycin (15 mg /kg b.wt)	3.04 ± 0.10^a	6.12 ± 0.44^a	2.24 ± 0.13^a	2.24 ± 0.11^a
BRCAE (250mg/kg b.wt) +Bleomycin	4.97 ± 0.22^{ab}	9.08 ± 0.39^b	4.85 ±0.18^{ab}	1.96 ± 0.04^b
BRCAE (500mg/kg b.wt) +Bleomycin	7.12 ± 0.35^b	14.96 ± 0.64^b	5.01 ± 0.28^{ab}	1.70 ± 0.07^b
Vitamin C (250mg/kgb.wt)+Bleomycin	6.7 ± 0.51^b	15.58 ± 0.79^b	5.05 ± 0.33^{ab}	1.73 ± 0.06^b

Group I- Control Group II- Bleomycin (15 mg /kg b.wt) Group III- BRCAE (250 mg/kg b.wt) + Bleomycin, Group IV- BRCAE (500 mg/kg b.wt) + Bleomycin Group V- Vitamin C (250 mg/kg b.wt) + Bleomycin, Values are expressed as mean ± SD for six animals. Group comparison and statistical significance at $p<0.05$: ^{a}: Group I vs. II, III, IV, V $^{b:}$ Group II vs. I, III, IV, V

The effect of BRCAE on the activity of the antioxidant enzymes (SOD and CAT), pulmonary GSH content and lipid peroxidation levels are presented in **Table 2**. A significant reduction in the activity of SOD, CAT and GSH were observed in the group II animals that served as BLM control animals. Lipid peroxidation levels as MDA content was observed to be markedly ($p<0.05$) elevated in the group II animals. Treatment with BRCAE at 250 mg/kg b. wt and 500 mg/kg b.wt to the animals of group III and IV respectively resulted in a marked improvement in the activity of SOD and CAT and a significant ($p<0.05$) raise in the levels of GSH with a marked decline in MDA content. Group V animals that were supplemented with the standard, vitamin C effectively normalized ($p<0.05$) the activity of the enzymes- SOD and CAT, increased the pulmonary GSH content and depressed the MDA levels. The results of the histopathological analysis of the pulmonary tissue of experimental animals are presented in **figure 1 (a-e)**. **Figure 1a** - represents the lung sectioning of the group I animals. Tissue presents normal lobular architecture with normal appearance and no obvious abnormality. **Figure 1b** – presents the pulmonary tissue of the group II animals that served as bleomycin control. Severe hemorrhage and necrosis was observed. Tissue representing intense scarring; features of inflammation**. Figure 1c**- The lung tissue sectioning of the group III animals treated with 250 mg/kg b.wt BRCAE. The tissue presents mild necrosis and milder infiltration of inflammatory cells. **Figure 1d**- The slide represents the lung section of the group IV animals treated with 500 mg/kg b.wt BRCAE. The sectioning reveals absence of necrosis and considerable reversal to normal architecture and very mild inflammation. **Figure 1e**- The lung tissue sectioning of the standard, vitamin C treated group V animals. The section presents mild patchy inflammations.

Histopathological analysis

Fig. 1a: Group I (Control)

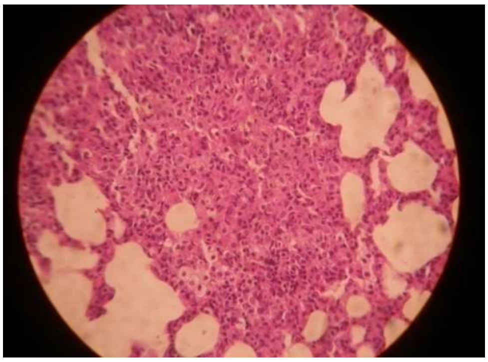

Fig. 1b: Group II (Bleomycin control)

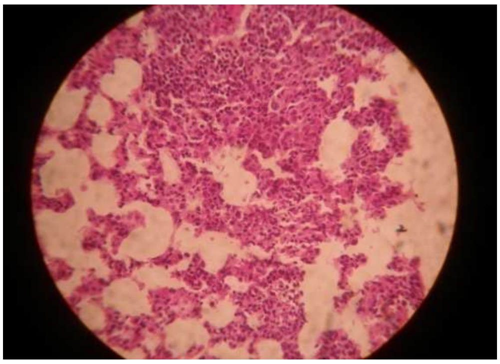

Fig. 1c: Group III

(250 mg/kg b.wt BRCAE+ Bleomycin)

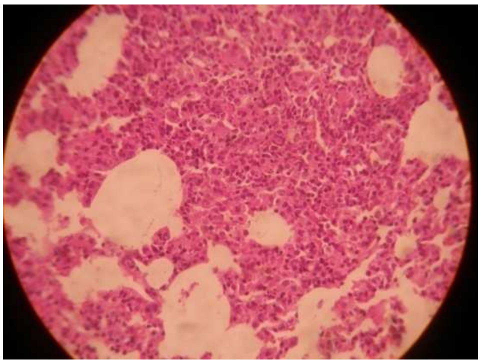

Fig. 1d: Group IV

(500 mg/ kg b.wt BRCAE+Bleomycin)

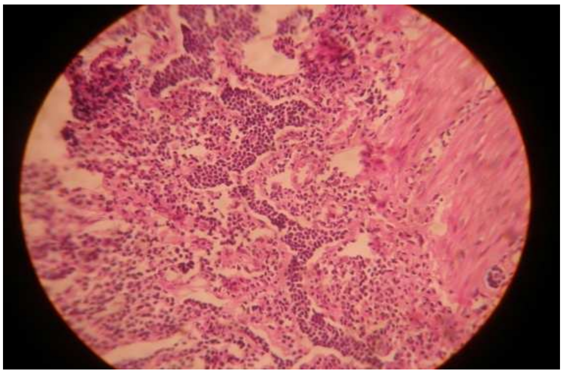

Fig. 1e: Group V

(Vitamin C 250 mg/kg b.wt +Bleomycin)

DISCUSSION

Idiopathic pulmonary fibrosis is a chronic diffuse interstitial lung disease characterized by failure of alveolar re-epithelialization, persistence of fibroblasts/myo-fibroblasts, and deposition of extra cellular matrix (ECM) and distortion of lung architecture. Glycoprotein comprises the connective tissue component of the ECM and plays a vital role in the pathogenesis of pulmonary fibrosis. Natural antioxidants, such as polyphenols from green tea extracts are known for their capability of reducing the expression of ECM genes [31].

Glycoproteins are predominantly protein in nature with one or more heterosaccharide chains that contains hexose, hexosamine, sialic acid and fucose. Many substances of biologic importance, including enzymes, hormones, antibodies and membranes, represent these conjugated proteins.

The elevation in the levels of glycoprotein components is due to the secretion of cell membrane glycoconjugates into the circulation[32]. The observed increase in the levels of glycoprotein moieties in BLM induced rats may also be due to increased deposition of macromolecular components, which is a physiological adjustment to the pathological process. Alterations in glycoprotein concentration during inflammatory conditions, lung fibrosis, malignancy, and in tumorous human lung tissue are reported earlier [33,34]. In addition, abnormal increase of glycoprotein components has been reported during pulmonary fibrosis [35]. Evidence has also been presented that pulmonary elastin and hexosamine contents are increased in BLM-injured lung tissue [36]. The observed increase in the sugar moieties of the glycoproteins can also be related to the augmentation in the concentration and synthesis of corresponding glycoprotein synthesizing enzymes.

Elevated levels of glycoprotein synthesizing enzymes are associated to the inflamed state and increased activities of sialyl and galactosyl transferase were observed in the serum and lung of inflamed rats during earlier studies [37]. Earlier reports suggest that during BLM administration, a variety of cytokines elaborated by the inflammatory cells may contribute significantly to the lung inflammation and may initiate the fibrotic process.

BLM increased the levels of glycoprotein moieties in the plasma. Supplementation with epigallocatechin-3-gallate (EGCG) reduced the levels of glycoprotein moieties in lungs and serum of BLM - induced rats, indicating its potential anti-inflammatory and immunosuppressive activity [38,39] that might have inhibited the inflammation and synthesis of glycoprotein synthesizing enzymes. Thus the observed reduction in the levels of hexose, hexosamine and sialic acid in the BRCAE treated animals suggests the protective effect of the extract. Living tissues are endowed with innate antioxidant defense mechanisms, such as the presence of the enzymes catalase (CAT), superoxide dismutase (SOD). A reduction in the activities of these enzymes is associated with the accumulation of highly reactive free radicals, leading to deleterious effects such as loss of integrity and function of cell membranes [40,41].

The activity of the antioxidant enzymes were observed to be decreased significantly ($p<0.05$) in the lung tissue of the animals that were induced with BLM as compared to group I animals. The administration of BRCAE to the animals of group III and group IV resulted in significant ($p<0.05$) improvement in the activities of the antioxidant enzymes ($p<0.05$) in a dose dependent manner as against the group II animals. A significant increase in the enzyme activities were observed in group V animals that were pretreated with standard vitamin C. The decreased levels of antioxidant viz., SOD and CAT activities may be due, in part, to an overwhelming oxidative modification of the enzymatic proteins by excessive ROS generation. More so, reduction in the activities of these enzymes may stem from decrease in their rate of synthesis.

The significant decrease in SOD activity due to BLM indicates inefficient scavenging of reactive oxygen species (ROS) which might be implicated to oxidative inactivation of enzymes. CAT is a key component of the antioxidant defense system. Inhibition of this protective mechanism results in enhanced sensitivity to free radical induced cellular damage. Administration of BRCAE increases the activities of CAT in BLM induced rat to prevent the accumulation of excessive free radicals and protects the lung from BLM induced toxication.

Houttuynia cordata extract was found to improve the activity of SOD and CAT in the lung tissue of the animals that were induced with BLM [42].

In addition, the GSH antioxidant system plays a fundamental role in cellular defense against reactive free radicals and other oxidant species. *Ginkgo biloba* extract was found to increase the activities of antioxidant enzymes in BLM induced pulmonary fibrosis [43]. Boswellic acid treatment was found to improve the activity of the antioxidant enzymes in the rats that were induced with BLM [44].

Thus from the results of the present study the improvement in the activities of the antioxidant enzymes could be due to the radical scavenging action of BRCAE.

Imbalance between oxidant and antioxidant defense mechanisms may contribute to incidence of pulmonary fibrosis [45]. Glutathione (GSH) is an intracellular thiol present in all tissues, including lungs. GSH acts as a non-enzymatic antioxidant that reduces H_2O_2, hydroperoxides (ROOH) and xenobiotic toxicity [46]. GSH provides a protection to the lung from oxidative damage induced by endogenous or exogenous lung toxicants [47]. However, its depletion in the lung by a fibrogenic agent, bleomycin as shown in the present study was associated with the risk of lung damage [[48,19],and[23]].

Co-administration of BRCAE with BLM reversed reduced glutathione depletion and subsequent lung damage. The ability of BRCAE to prevent depletion of lung glutathione stores suggests that its antifibrotic activity in the BLM model is mediated at least in part by its antioxidant properties. BRCAE may save intracellular reduced glutathione by acting as either ROS scavenger or by enhancing GSH synthesis.

GSH acts synergistically with vitamin E in inhibiting oxidative stress and acts against lipid peroxidation [49]. Vitamin-C also scavenges and detoxifies free radicals in combination with vitamin-E and glutathione [50]. It plays a vital role by regenerating the reduced form of vitamin-E and preventing the formation of excessive free radicals [51].

The results of our study were in accordance with the previous studies with antioxidants. Alpha- lipoic acid was found to improve the levels of antioxidants in BLM induced rats [52]. El-Medany et al [23]reported, treatment with mesna to the BLM induced rats improved the levels of GSH. Sener et al [53]reported that resveratrol alleviated the BLM induced lung fibrosis. Resveratrol was found to improve the levels of GSH in the lung tissue that was depressed on treatment with BLM. Boswellic acid was found to improve the levels of GSH content in BLM induced rats [44].

The present study shows that after BLM treatment the ratio of lipid peroxidation was increased as a result of the production of malondialdehyde (MDA) the above result meet the reported data that demonstrate apparent elevation in lung MDA after the administration of BLM. The decrease in the TBARS levels by co administration of BRCAE at 250 mg/kg b.wt and 500 mg/kg b.wt suggest the capacity of the extract in combating the effect of BLM induced toxicity.

Ng et al [42] reported that *Houttuynia cordata* extract was able to reduce the levels of MDA that was elevated upon BLM induction, suggesting the protective effect of the extract.

Protein carbonyl and thiobarbituric acid reactive substances levels, which were significantly elevated in the bleomycin treated rats, were significantly attenuated by melatonin was reported by Yildrim et al [54]. Alpha-Lipoic acid treatment was found to depress the levels of MDA in BLM induction in rats [52]. Iraz et al [43] reported a significant increase in MDA levels in the lung tissue of the rats that were induced with BLM. Boswellic acid was reported by Ali and Mansour [44] to improve the antioxidant status and reduce the levels of MDA.

Thus by improving the activities of the antioxidant enzymes and GSH levels, and decreasing MDA, BRCAE was found to ameliorate the effects of BLM and offered protection. The protective effects of the extract of bokchoy could be attributed to the phytochemicals harbored as revealed in the previous in vitro analysis.

CONCLUSION

The results observed thus suggest the bokchoy extract at both doses (250 mg/kg b.wt and 500 mg/kg b.wt) effectively ameliorated the toxic effect of bleomycin in a dose dependent manner. Thus suggesting the extract for further exploitation in the field of combating side effects of chemotherapeutic drugs.

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