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Bleomycin-induced pulmonary toxicopathological changes in rats and its prevention by walnut extract

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ABSTRACT

Oxidative stress-related inflammation and apoptosis are important pathogenic consequences, which result in acute pulmonary toxicity. Bleomycin (BLM) is used to treat various forms of cancers. However, its prolonged administration is associated with major toxicity to respiratory system. We studied the effect of walnut (*Juglans regia)* extract in a rat model of BLM-induced pulmonary toxicopathy. We also studied parameters of inflammation, apoptosis and oxidative stress in various groups of animals. Prophylactic treatment of total methanolic extract of walnut at the dose of 150 mg/kg b.w. was given *per os* to Wistar rats for 14 days prior to BLM exposure. A single intratracheal injection of BLM (10 U/kg b.w.) was administered on the eleventh day of the treatment. There was a marked increase in the hydroxyproline level, lipid peroxidation, nitric oxide production, and in the activities of xanthine oxidase and myeloperoxidase in the lung tissue in BLM-treated animals when compared to control animals. BLM also decreased the activities of antioxidant enzymes such as glutathione reductase and catalase and increased the lung inflammation and apoptosis by upregulating the NF-κB signaling pathway and caspase-3 expression. Treatment with walnut extract attenuated these changes in a significant manner. Walnut extract significantly modulated the lung injury as measured by markers of cellular injury such as lactate dehydrogenase and alkaline phosphatase, total cell count, total protein and reduced glutathione in bronchoalveolar lavage fluid. Histological findings supported the protective effects of walnut extract against BLM-induced lung injury. Walnut which has been shown to have numerous medicinally valuable constituents including ellagic acid showed efficacy in preventing the various toxicopathological effects of BLM in rat lungs. Overall, walnut extract decreases BLM-induced oxidative stress and lung inflammation by modulating the alveolar macrophage inflammatory response in rats and thus protecting them from the pathological effect of BLM.

1. Introduction

Antineoplastic drugs disrupt oxidant-antioxidant balance in the lungs [1–3]. These drugs cause pulmonary toxicity due to their direct drug action or synergistic or antagonistic action of two drugs [4]. Different groups of antineoplastic drugs at high doses cause massive hepatotoxicity, nephrotoxicity, and pulmonary toxicity [5–7]. Bleomycin (BLM) a glycopeptidic antibiotic is an effective antineoplastic drug used in the treatment of breast cancer, ovarian cancer, lung cancer, and different types of leukaemia [8]. Its toxicity leads to the generation of reactive oxygen species (ROS) and lipid peroxide formation [9]. Such effect at the cellular level contributes to the pathogenesis caused by acute and chronic pulmonary toxicity by BLM [10,11]. The effective use of BLM in chemotherapy remains limited, since it precipitates dose dependent interstitial pneumonitis that often progresses to interstitial pulmonary fibrosis [12]. BLM administration results in increased lipid peroxidation (LPO) and altered activities of antioxidant enzymes in bronchoalveolar lavage fluid (BALF) and lung tissue [13,14]. Intratracheal administration of BLM decreases antioxidants and generates reactive oxygen metabolites including superoxide and hydroxyl radicals [15]. These metabolites are responsible for the DNA damage, LPO, and disturbance in lung prostaglandin synthesis and an increase in lung collagen formation [15]. The development of pulmonary fibrosis is associated with and likely preceded by an increase in the influx of activated

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inflammatory cells within the lung parenchyma [16]. The cells under inflammation produce ROS that may contribute to the pathogenesis of BLM-induced lung injury [15]. Increased ROS in turn provoke inflammatory responses resulting in the release of proinflammatory cytokines and chemokines [17]. Drug and chemically- induced inflammation can lead to the expression of proinflammatory proteins such as NF-κB which is overexpressed in case of lung inflammation [18]. NFκB regulates the expression of cytokines, inducible nitric oxide synthase (iNOS), cyclo-oxgenase 2 (COX-2), growth factors, inhibitors of apoptosis and effector enzymes in response to ligation of many receptors involved in immune function e.g., T-cell receptors (TCRs), B-cell receptors (BCRs) and IL-1 receptor superfamily [19,20]. Moreover, pathological dysregulation of NFκB is linked to inflammatory and autoimmune diseases and cancer [21]. BLM and cyclophosphamide (CP) exposure causes the release of various cytokines that modulate inflammatory responses [22,23]. ROS also alleviate apoptosis by inducing the activation of caspases. Caspase-3 is an important caspase protein and apoptotic effector which leads to a cytoskeletal breakdown, nuclear demise, and other cell changes associated with apoptosis [24]. Apoptosis has also been involved in several pulmonary disorders caused by drugs [25]. The induction of apoptosis by other pneumotoxic agents such as silica [25], immune complexes [26,27] and endotoxin [28] has been associated with lung pathologies including lung injury, pulmonary inflammation and fibrosis.

ROS mediated damage can be prevented by a number of herbal extracts [29] and natural agents [30]. Herbal extracts of *Asparagus racemosus*, *Cassia occidentalis*, *Emblica officinalis,* and *Withania somnifera* reduce the toxicity of conventional chemotherapeutic drugs [31–33]. Anticancer drugs have often been used in combination with potential protective agents for eliminating their toxic effects [34]. Natural products not only prevent BLM induced toxicity but also stimulate the antioxidant profile of the affected tissues [35,36].

Among various natural agents walnut (*Juglans regia* L; family: Juglandaceae) extracts have shown potential to reduce toxicity of antineoplastic agents because of its antioxidant properties [37–39]. Walnut has been used in several folklore and traditional systems of medicine world over [40]. It has been reported that the antioxidant property of *J. regia* bark extract protects the urinary bladder against the toxicity of CP in mice [38] and the leaf extracts of walnut are strong scavengers of pro-oxidant reactive species [41,42]. Studies have been reported on the protective effect of *Houttuynia cordata* [43] and *Ginkgo biloba* [44] against BLM-induced pulmonary fibrosis.

The available data indicate that *J. regia* has promising antioxidant and toxicity preventing activities against toxicants. Ellagic acid (EA) is a principal active dietary polyphenolic constituent present in walnut extract (WE). It is pharmacologically active as an anti-inflammatory, antioxidant and antitumor agent [45]. The present study was executed to test the antioxidant potential of WE against BLM-induced pulmonary pathogenic changes, oxidative stress, inflammatory onslaught and apoptosis. We also explored the protective effect of total extract of *J. regia* against BLM-induced pulmonary pathogenic changes.

2. Materials and methods

2.1. Bleomycin and plant extract

BLM was purchased from Khandelwal Laboratories, New Delhi, India. BLM was provided in a white crystalline form. The total methanolic extract of *J. regia* in semisolid form was purchased from the Plant Extract Division of Saiba Industries Pvt. Ltd, Mumbai (Batch No. C/2727). The walnut extract was a dark brown, thick paste having a medicinal odour and taste. The moisture content of the extract was 13% as measured by Karl Fischer method. The ash content of the extract was 4.4%. The extract was endotoxin free as certified by the manufacturers.

2.2. Phytochemical analysis of extract for EA content

Ellagic acid content in the extract was measured using CAMAG HPTLC Scanner III apparatus with the help of Wincats software. A 1 mg/ml stock solution of standard (purity >90%) was prepared by taking accurately 5 mg of EA into 5 ml volumetric flask and 3 ml mixture of methanol and water (80:20 v/v) was added and sonicated for 5 min to dissolve it completely. Subsequently, 2 gm of sample was taken into 25 ml volumetric flask and 20 ml of aqueous acidic alcohol solution (5% HCL in 80:20 v/v) was added to the sample and it was sonicated for 20 min in hot conditions. Volume was adjusted with the same solvent and filtered. The filtered sample was used for the analysis and 5 ml of the filtered sample was mixed with equal amount of chloroform. The upper layer (polar) was separated and the amount of EA in the sample was estimated. The standard solution of 0.4 μl and sample with three concentrations of 6, 3 and 4 μl was applied onto 5 × 10 cm precoated silica plate in duplicate. The solvent system toluene: ethyl acetate: formic acid: methanol (30:30:8:3 v/v/v/v) was used as mobile phase. The plate was developed by spraying anisaldehyde-sulfuric acid reagent and kept at 110 °C for 10 min and scanned at 600 nm.

2.3. Animals

The study was conducted in male Wistar rats (body weight of 150 ± 30 gm) provided by the Central Animal House Facility of the University. Study protocols were approved by the Institutional Animal Ethics Committee of the University (IAEC; Project # 696). The animals were maintained under standard laboratory conditions (temperature 25 ± 2 °C; photoperiod of 12 h). Commercial pellet diet and water were given *ad libitum*.

2.4. Treatment

Bleomycin and walnut extract were dissolved in normal saline (0.9% NaCl). Before each administration, solutions were thoroughly vortexed to obtain a homogenous suspension. Bleomycin was administered intratracheally (*i.t.*) at the dose of 10 U/kg b.w. walnut extract was given orally (*per os*) at the dose of 150 mg/kg b.w. The dose volume for BLM and walnut extract was 0.3 ml and 2 ml per kg b.w. respectively. The doses and schedule of treatment of BLM and walnut extract have been based on the previous studies [38,45,46]. Rats were divided into four groups ($n = 6$). The animals of group I served as control and received normal saline orally for 14 days and a single dose of normal saline by *i.t.* route on the eleventh day of the study. Group II animals were administered with a single dose of BLM on the eleventh day of the treatment schedule. Group III received walnut extract *per os* once daily for 14 days. Group IV received a single dose of BLM on the eleventh day of the treatment schedule along with daily administration of walnut extract for 14 days.

2.5. Collection of bronchoalveolar lavage fluid (BALF)

Rats were sacrificed under mild anaesthesia with chloral hydrate (Sigma–Aldrich Co., St. Louis, MO, USA) on day 15 and a tracheotomy was performed. Ice-cold phosphate buffer saline (PBS) (0.5 ml) was instilled into the lungs and BALF was obtained by aspirating three times using tracheal cannulation. The BALF was centrifuged and the supernatant was collected and stored at