Induction of heme oxygenase-1 attenuates chemotherapy-induced pulmonary toxicity in rats: A possible link between heme oxygenase-1 and NF-κB

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Abstract: A critical restriction in the use of bleomycin (BLM) is development of pulmonary fibrosis via oxidative and inflammatory mechanisms. Drugs that induce heme oxygenase-1 (HO-1) like hemin (HEM), have anti-inflammatory, antioxidant, and immunomodulatory effects. Accordingly, it is worth to test HEM against BLM-induced lung Injury. Four groups of rats were used: control group; HEM group (50mg/kg, i.p.); BLM group (5mg/kg, intratracheal single injection) and HEM+BLM group (HEM administered 1 day before BLM injection and continued for 14 days). At the end of experiment, lactate dehydrogenase (LDH) and NO levels were estimated in bronchoalveolar lavage fluid (BALF). Hydroxyproline (HP), myeloperoxidase (MPO), IL-6, GSH, MDA levels and SOD activity were determined in lung tissues. In addition, expression of HO-1 and NF-κB protein in lung tissues was determined using both western blot and immunohistochemical techniques. Also lung tissues were investigated histopathologically. BLM produced lung damage as indicated from the elevation in LDH and NO, perturbation in lung oxidative stress indicators, increased HP, MPO, IL-6 contents and NF-κB expression. On the other side, HEM, reduced BLM harmful effects as noticed from amelioration of biochemical markers and histopathological lesions, which is concomitant with over-expression of HO-1. Therefore, induction of HO-1 in lung by HEM may alleviate the lung damaging effects of BLM.

Keywords: Bleomycin, hemin, HO-1, lung tissue, hydroxyproline, IL-6, NF-κB.

INTRODUCTION

Pulmonary fibrosis is a progressive lung disease, which eventually leads to respiratory failure. After diagnosis, patients are expected to live only two-six years (Selman *et al.,* 2001). Among the causes of pulmonary fibrosis is exposure to the chemotherapeutic bleomycin (Zhao *et al.,* 2014). Currently, the *in vivo* animal studies using bleomycin-induced pulmonary fibrosis model is utilized to understand the pathogenesis of pulmonary fibrosis **(**Moore and Hogaboam, 2008). Bleomycin (BLM) is anticancer antibiotic, formed by the bacterium "Streptomyces verticillus" (Azambuja *et al.,* 2005). It is used mainly in treatment of testicular carcinoma, Hodgkin and non-Hodgkin lymphomas (Bugaut *et al.,* 2013). A serious limitation in the use of bleomycin during chemotherapy is the incidence of dose-dependent pulmonary fibrosis in human and experimental animals (Kim *et al.,* 2010). Injection of bleomycin to animals produced pulmonary fibrosis, which is similar histologically and physiologically to human chronic fibrotic lung disease. This observation led to the development of an animal model in which bleomycin at a single dose induced histopathological changes resembling human idiopathic pulmonary fibrosis (Lazo *et al.,* 1990). Many mechanisms are involved in bleomycin-induced pulmonary injury and fibrosis. Upon binding to DNA and iron, bleomycin induces free radicals and inflammatory alterations which contribute to DNA damage and collagen accumulation in the lung (Kim *et al.,* 2010). Moreover, BLM produces a deficiency in internal antioxidant defenses thus intensifying oxidative tissue damage (Atzori *et al.,* 2004).

Heme oxygenase-1 (HO-1) is the rate-limiting enzyme in heme catabolism. It is induced by free radical-initiated reactions, and considered to be an adaptive response against oxidative tissue damage (Fouad *et al.,* 2009). In addition, HO-1 is reported to exhibit powerful anti-inflammatory and immunomodulatory effects (Origassa and Camara, 2013). Previous studies have shown that HO-1-inducing agents, as hemin, can alleviate nephrotoxic effects caused by a wide array of stressors, including mercury (Yoneya *et al.,* 2000). Based on the previous data, the present study aimed to examine whether HO-1 is expressed in the lung tissues by immunohistochemical technique and, if so, to determine if the activation of HO-1 (by hemin) would have protective effects against BLM induced lung toxicity in rats. For this purpose, we have evaluated the level of lactate dehydrogenase (LDH) and NO (as nitrite/nitrate) levels in BALF. Moreover, lung tissues hydroxyproline (HP), myeloperoxidase (MPO), IL-6 levels, HO-1, NF-κB expression (by immunohistochemical/western blot analysis) were evaluated. In addition oxidative stress markers (GSH, MDA and SOD) were also estimated in

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lung tissues. Histopathological evaluation was also performed on lung tissues using hematoxylin & eosin (H&E) stain in addition to Masson trichrom stain for collagen deposition and fibrosis detection.

MATERIALS AND METHODS

Drugs and chemicals

Bleomycin hydrochloride (Bleocin, Nippon Kayaku Co., Ltd., Tokyo, Japan). Hemin was obtained from Sigma Chemical Co., USA. All other chemicals used were of good quality and analytical grade.

Animals

Adult Wistar albino female rats (180-200g) were purchased from the national research center, Egypt. Standard diet and water were given to animals ad-libitum. Rats were housed under conventional conditions for a week before treatment. All animal groups were of the same sex (female) to exclude sex-difference effect on the results. Institutional guidelines for ethical care of animals were followed for all experiments. The study protocol was approved by research ethics committee (NO 3/2013), Faculty of Pharmacy, Damanhour University, Egypt.

Experimental protocol

Rats were randomized into four groups (6 animals /group). After recording the body weights, the rats were anesthetized. Exposing proximal portion of the trachea by blunt dissection of the overlying skin.

Group I: Control (CO) group: animals were injected intratracheally only once with sterile 0.9%. saline.

Group II: hemin (HEM) group: animals received hemin (50 mg/kg/day, i.p.) (Desbuards *et al.,* 2007) one day before a single intratracheal saline injection and continued for 14 days.

Group III: bleomycin (BLM) group; following anesthesia, a single intratracheal injection of BLM (5 mg/kg) was administered to rats (Jin *et al.,* 2014). We chose to administer bleomycin intratracheally because previous studies showed that a single intratracheal dose is sufficient to produce marked histological and biochemical changes in most rodents within 2~4 weeks (Zhou *et al.,* 2007; Kilic *et al.,* 2014) .

Group IV: both drugs (HEM+BLM)-treated group; hemin (50 mg/kg, i.p.) was administered one day before the intratracheal BLM injection and continued for 14 days. This time course (14 days) was selected based on many studies, which adapted this period as most of biochemical parameters are still evident during this time-course. Also, histological data revealed that at 14 days lung parenchymal inflammation is present and a not very well established fibrosis (Sangiuolo *et al.,* 2013; Kilic *et al.,* 2014) .

Bronchoalveolar lavage fluid (BALF) and lung tissues preparation

Twenty-four hours after the last dose of the specific treatment, all rats were weighed and anesthetized. Then, the lung and the trachea were exposed after dissecting of chest cavity, the tracheae were cannulated and the lungs were lavaged five times. Through needle hub, cooled saline was administered and aspirated slowly. The bronchoalveolar lavage fluids were centrifuged for 10 min at 1500 rpm. The supernatants were stored at -80°C for a later determination of LDH and NO (Lim and Chung, 2014). The lung lobes were isolated, weighted for lung/body weight ratio estimation. Then lung lobes were washed in ice-cold saline, and then quickly stored at -80 °C for subsequent procedures.

Biochemical evaluation

Determination of lactate dehydrogenase (LDH) activity in BALF

LDH activity was determined in BALF by commercially available LDH kit (Linear Chemicals, S.L., Spain) following the method of Whitaker (1969). Using this method, LDH catalyses the reduction of pyruvate to lactate in the presence of reduced NADH at pH 7.5. The rate of decrease in absorbance at 340 nm resulting from the oxidation of NADH to NAD^+ was measured which is proportional to the activity of LDH present in the sample.

Measurement of NO contents in BALF

Nitric oxide was estimated in BALF by commercially available NO assay kit (Biodiagnostic, Dokki, Giza, Egypt) according to the method of Montgomery and Dymock (1961). Principle: In acidic medium and in the presence of nitrite the formed nitrous acid diazotizes sulphanilamide and the product is coupled with N-(1–naphthyl) ethylenediamine. The bright reddish-purple color of the azo dye can be measured at 540 nm .

Preparation of lung tissues homogenates

Lung samples were thawed and homogenized (10% w/v) in ice-cold 0.1M Tris-HCl buffer (pH 7.5) for 15 min. Homogenates were filtered and centrifuged by using a refrigerated centrifuge at 4°C (Dengiz *et al.,* 2007)**.** The supernatants were used for determination of hydroxyproline, MPO, IL-6 and oxidative stress biomarkers.

Determination of lung tissues hydroxyproline (HP) contents

Lung HP content was determined as a biochemical index of parenchymal collagen content. HP lung tissues content was determined using rat hydroxyproline ELISA kit (Mybiosource, San Diego, CA, USA) according to the manufacturer's protocol.

Estimation of myeloperoxidase (MPO) levels in lung tissues

Rat MPO ELISA kit, (Wkea Med Supplies, China) was used to estimate MPO levels in lung tissues, according to the manufacturer's protocol.

Determination of IL-6 contents in lung tissues

Rat IL-6 ELISA kit, (Wkea Med Supplies, China) was utilized to estimate IL-6 levels in lung tissues, according to the manufacturer's protocol.

Estimation of reduced glutathione (GSH), malondialdehyde (MDA) levels and superoxide dismutase (SOD) activity in lung tissues

Lung tissue homogenate was utilized to determine GSH content using Ellman's reagent (Ellman, 1959). MDA used as lipid peroxidation indicator. MDA was determined in rat lung homogenates following the method of Mihara and Uchiyama (1978)**.** SOD activity was also determined according to the method of Marklund (1985).

Immunohistochemical detection of HO-1 and NF-κB in lung tissues

Formalin fixed paraffin sections were processed for immunohistochemical detection of HO-1 and NF-κB in lung tissues. Mouse monoclonal anti-HO-1 and anti-NF-κB (Santa Cruz Biotechnology, Inc**)** were used as a primary antibodies which linked with biotinylated goat anti-mouse lgG antibody (Dako, LASB Universal). The percentages of positive cells stained for HO-1 and NF-κB per field in each experimental group were determined after comparison with control sections.

Western blotting technique for HO-1 and NF-kB protein expression in lung tissue

Briefly, each sample homogenate from lung was processed for detection of HO-1 and NF-κB protein expression using mouse monoclonal anti-HO-1, anti-NF-κB or mouse monoclonal anti-actin (Santa Cruz Biotechnology, Inc.). Relative intensities of protein bands were analysed by scanner and quantified by AIDA Image Analyzer software

Histological studies

Autopsy samples were taken from the lung of rats in different groups and fixed in 10% formol saline then processed for staining by hematoxylin & eosin stain and Masson trichrom for collagen then examination was done through the light electric microscope (Banchroft *et al.,* 1996).

STATISTICAL ANALYSIS

Data analysis was achieved using Graph Pad Prism version 6.00 (Graph Pad Software, San Diego, CA, USA). Results were expressed as the mean ± standard errors. Statistically significant difference was determined by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. Probability values (P) less than 0.05 were considered to be statistically significant

RESULTS

Effects of hemin and/or bleomycin on body weight, wet lung weight and lung/final body weight ratio

BLM significantly reduced body-weight, and increased lung-weight of rats and consequently the lung/body weight ratio increased also versus control. HEM administration antagonized only BLM-induced decline in body-weight and improved lung/body weight ratio (table 1).

LDH and NO levels in BALF

LDH enhanced by 112% after BLM compared to control level. In addition, NO was increased by 109% after BLM administration in BALF. Pretreatment with HEM before and after BLM resulted in a marked reduction in LDH (33.96%) and NO (47%) levels compared to BLM-treated groups (fig. 1a, b).

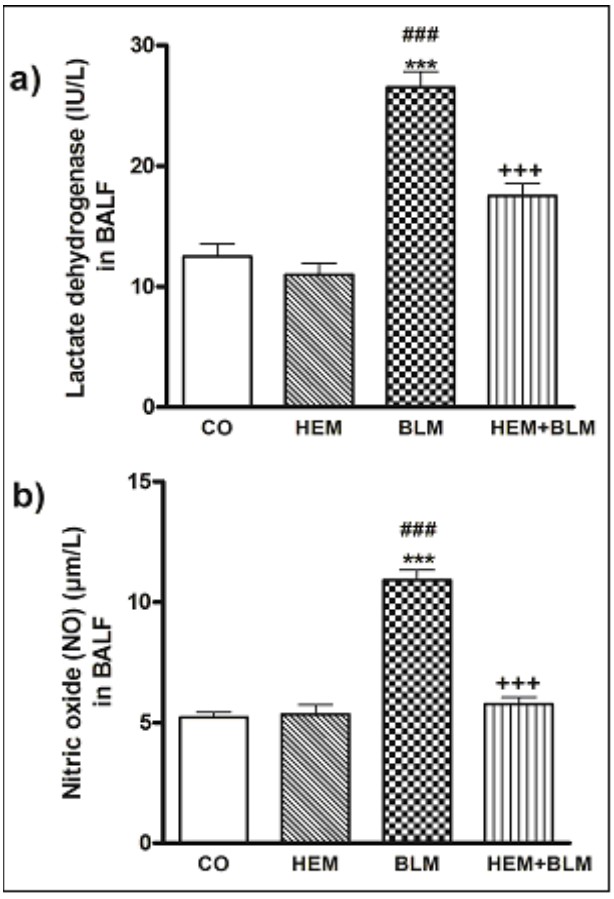

Fig. 1: Effects of hemin (HEM), bleomycin (BLM) and their combination (HEM+BLM) on LDH (a), NO (b) levels in BALF of rats compared to control (CO) group. The significant difference between two groups was determined by ANOVA followed by Tukey's multiple comparison test. ***p<0.001: Statistically significant difference from CO group. $^{###}$p<0.001: Statistically significant difference from HEM group. $^{+++}$p<0.001: Statistically significant difference from BLM group.

Table 1: Effects of hemin, bleomycin and their combination on body weight, lung weight and lung/body weight ratio

Treatment	Initial body weight (g)	Final body weight (g)	Change in body-weight (g)	Wet weight of the lung (g)	Lung/final body weight ratio
Control	180±2.8	195±1.7	15±1.2	0.7±0.065	0.0035±0.00031
Hemin (HEM)	182±1.6	196±2.02	14±1.1	0.68±0.022	0.0034±0.00014
Bleomycin (BLM)	194±2.3	181±3.16	-13***±0.9	0.89±0.032	0.0049***±0.0001
Both (HEM + BLM)	193±2.1	190±3.86	-3$^{**,+}$±0.41	0.8±0.038	0.0042*±0.00012

The significant difference between two groups regarding changes in body-weight, wet weight of lung and lung/body weight ratio was determined by ANOVA followed by Tukey's multiple comparison test. *p<0.05; **p<0.01; ***p< 0.001: statistically significant difference from the control group.$^{+}$p<0.05: statistically significant difference from the bleomycin group.

Hydroxyproline (HP) contents in lung tissues

HP is used as an indicator of collagen formation and for assessment of fibrosis. The lung contents of HP (mg/g tissue) augmented by 534% after BLM administration versus control. Pretreatment with HEM before and after BLM treatment reduced the HP by 60% as against BLM group. Giving HEM alone did not produce any significant changes in HP (fig. 2a).

MPO levels in lung tissues

The lung contents of MPO (infiltration indicator) (ng/g tissue) was increased by 258% after BLM administration versus control. Administration of HEM before and after BLM treatment decreased the MPO level by 57% compared with BLM group. HEM alone did not induce any marked alterations in MPO levels as against control (fig. 2b).

IL-6 contents in lung tissues

BLM administration significantly increased lung tissue of the proinflammatory cytokine IL-6 (pg/ g tissue) by 170% versus control. HEM decreased the elevated IL-6 level by 43.43% versus BLM. When used alone, HEM did not induce any changes in IL-6 levels (fig. 2c).

GSH, MDA levels and SOD activity in lung tissues

BLM reduced GSH levels by 41% versus control. HEM antagonized BLM-induced decline in GSH content and increasing GSH by 63% as against BLM group (fig. 3a). BLM elevated MDA levels by 125% above normal control level. HEM attenuated the MDA levels by 33% (fig. 3b). The activity of SOD reduced in lung tissues of BLM-treated rats by 54.6% versus control. However, HEM administration augmented this reduced SOD activity by 89% (fig. 3c). Tissues from HEM-treated group showed no significant difference from control with regard to GSH, MDA levels or SOD activity (fig. 3a- c).

Immunohistochemical analysis of HO-1 and NF-κB in lung tissues

Mild immune staining reaction was obvious in lung tissues of control group. The activity of HO-1 and NF-κB stained respectively 10 and 12% of the field in lung. Section of lung of rats administered HEM revealed strong expression of HO-1 (60%) and mild expression of NF-κB

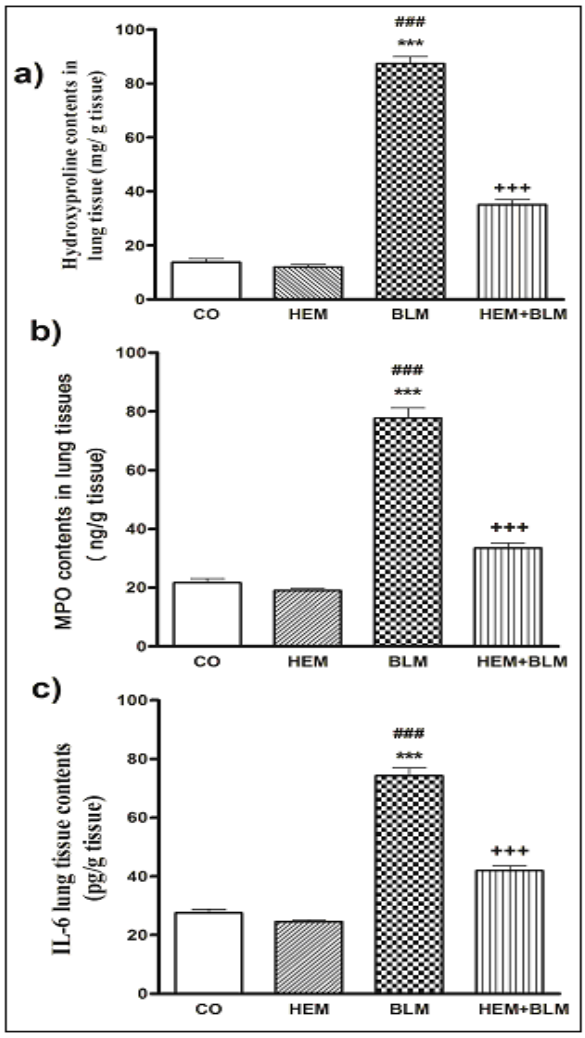

Fig. 2: Effects of hemin (HEM), bleomycin (BLM) and their combination (HEM+BLM) on hydroxyproline (a) myeloperixidase (MPO) (b) and IL-6 (c) contents in lung tissues of rats compared to control (CO) group. The significant difference between two groups was determined by ANOVA followed by Tukey's multiple comparison test. ***p<0.001: Statistically significant difference from CO group. $^{###}$p<0.001: Statistically significant difference from HEM group. $^{+++}$p<0.001: Statistically significant difference from BLM group.

(10%) (fig. 4a). Samples of lung of animals injected with BLM displaying strong NF-κB activity (75%) in the tissues. The positive cells stained for HO-1 was only 15% after BLM. Samples of lung tissues injected with both HEM and BLM showed strong expression of HO-1 (70%) and moderate expression of NF-κB (25%) (fig. 4a)

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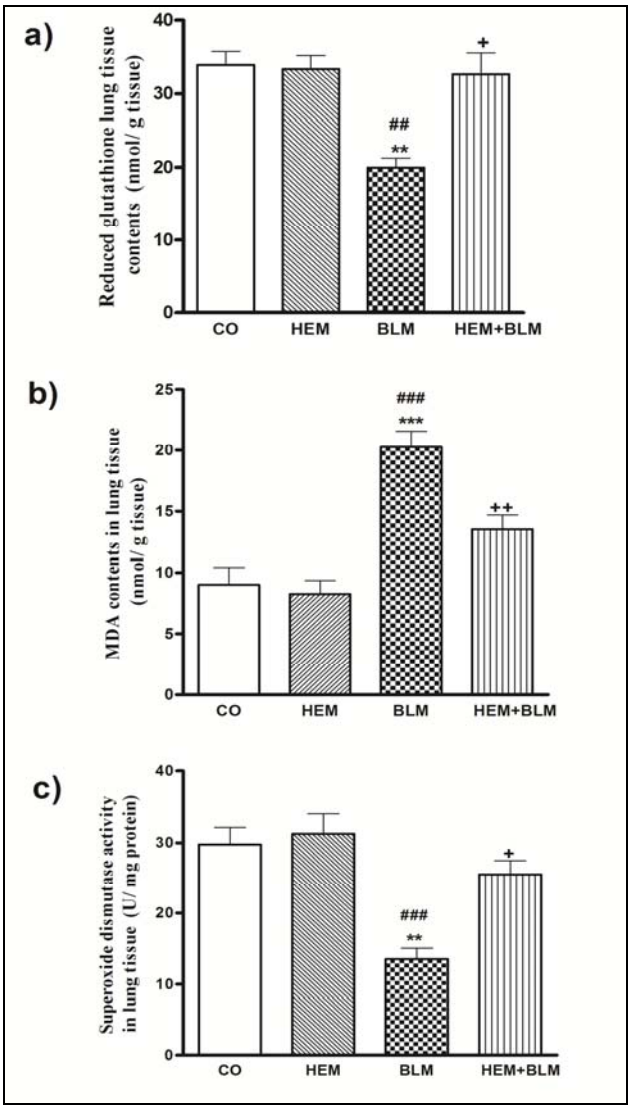

Fig. 3 Effects of hemin (HEM), bleomycin (BLM) and their combination (HEM+BLM) on oxidative stress biomarkers: glutathione (GSH) (a), malondialdehyde (MDA) levels (b), superoxide dismutase (SOD) activity (c) in lung tissue of rats compared to control (CO) group. The significant difference between two groups was determined by ANOVA followed by Tukey's multiple comparison tests. **p<0.01;***p<0.001: Statistically significant difference from CO group. $^{##}$p<0.01; $^{###}$p <0.001: Statistically significant difference from HEM group. $^{+}$p<0.05; $^{++}$p<0.01: Statistically significant difference from BLM group.

Western blot technique for HO-1 and NF-κB in lung tissues

There is significant over-expression of HO-1 protein in both HEM and HEM+BLM-treated rat tissues (fig. 4b). On the other hand, NF-κB expression increased after BLM treatment versus control as shown in fig. 4b. However, the protein expression of NF-κB was significantly reduced in group treated with HEM before BLM (fig. 4b).

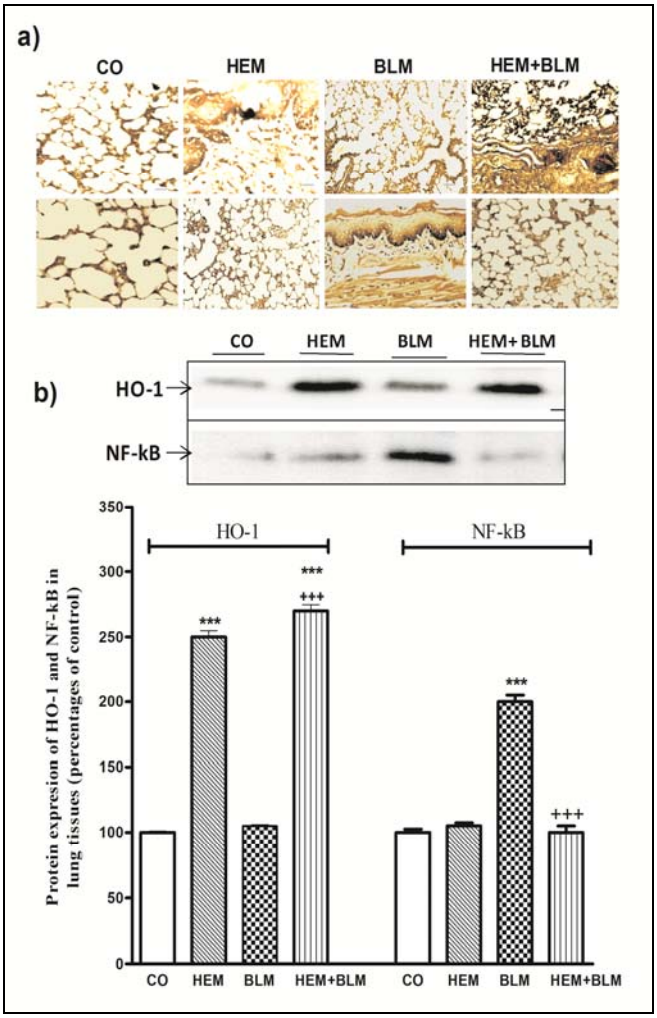

Fig. 4: Photographs showing the immunohistochemical staining of lung tissues for HO-1 and NF-kB in different groups: control (CO), hemin (HEM), bleomycin (BLM) and their combination (HEM+BLM) (a). Scale bar is 50 μm for all photos (a). Effects of HEM, BLM and their combination (HEM+BLM) on HO-1 and NF-kB protein expression in lung tissues using western blot technique (b). The significant difference between two groups was determined by ANOVA followed by Tukey's multiple comparison tests. ***p<0.001: Statistically significant difference from CO group. $^{+++}$p<0.001: Statistically significant difference from BLM group.

Morphological and histopathological evaluation of lung tissues of different experimental animal groups

Apparent inspection of lung tissues from animals kept as control showed normal appearance (fig. 5A). No histopathological lesions were noticed in bronchiole, air alveoli and blood vessels (fig. 5B, C). There was no morphological or histopathological alterations were recorded in rats treated with HEM (fig. 5D-F). Rats treated with BLM showed severe hemorrhage (fig. 5G), congestion in the blood vessels associated with focal fibrosis replacing the atrophied collapsed alveoli, hypertrophy in the musculature with formation of emphysematous air alveoli (fig. 5H). Focal fibrosis was detected by Masson trichrom stain as recorded in fig. 5I. Sections from lung of rats injected with HEM before and after BLM revealed normal morphology (fig. 5J) except for mild congestion in blood vessels (fig. 5K) with no fibrosis by Masson stain (fig. 5L). We attempted to compare between the lesions appeared in different groups and give them scores according to its severity by inspection of a minimum of 8 fields from each lung section by an observer blinded to the treatments. The results of histopathology analysis and alterations from normal control lung tissues were also displayed in table 2.

Table 2: The severity of histopathological alterations in lung of experimental groups of rats treated with hemin (HEM), bleomycin (BLM) and their combination (HEM+BLM) in comparison to control (CO) non-treated rats.

Histopahological Lesions	CO	HEM	BLM	HEM+BLM
Congestion	0	0	+++	+
Hemorrhage	0	0	+++	0
Alveolar emphysema	0	0	++	0
Muscular hypertrophy	0	0	++	0
Focal fibrosis	0	0	++	0

Data were obtained from investigation of four histological sections from the lung of control group (CO), HEM-treated group, BLM-treated group, and their combination (HEM + BLM). A minimum of 8 fields from each lung section were examined by an observer blinded to the treatments of the animals and assigned for severity of changes using scores of 0 (absent) and + (mild level): less than 25% of the total fields examined revealed histopathological alterations. ++ (moderate level): less than 50% of the total fields examined revealed histopathological alterations. +++ (severe level): less than 75% of the total fields examined revealed histopathological alterations

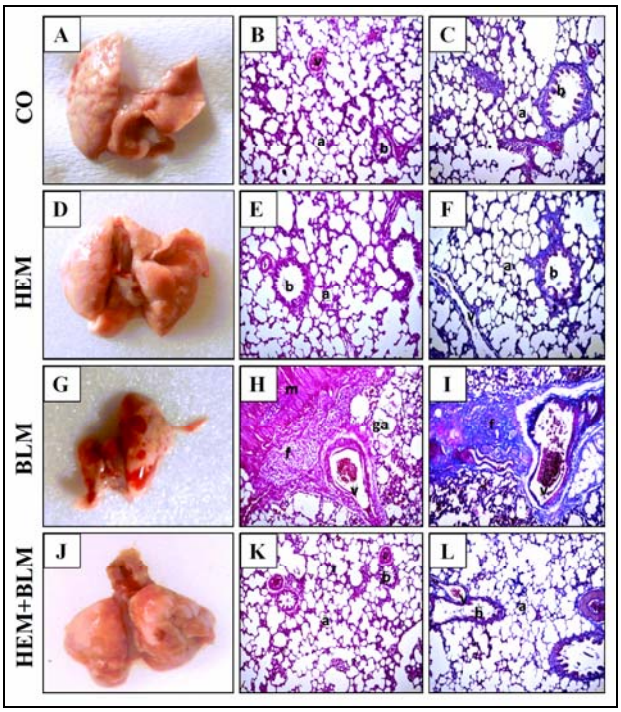

Fig. 5: Photographs showing the effects of hemin (HEM) against morphological and histopathological alterations induced by bleomycin (BLM) in the lung tissues. Apparent inspection of control lung tissues showed normal appearance (photo A). Normal histological structure of the bronchiole (b), air alveoli (a) and blood vessels (v) were shown in photos B, C [H&E and Masson trichrom (MT) x 16]. In rats treated with HEM, normal morphological and histological structure of the bronchiol (b), air alveoli (a) and blood vessels (v) were observed (Photos D-F) [H&E and Masson trichrom (MT) x16]. Rats treated with BLM showed sever hemorrhage (Photo G), congestion in the blood vessels (v) associated with focal fibrosis (f) replacing the atrophied collapsed alveoli, hypertrophy in the musculature (m) with formation of emphysematous giant air alveoli (ga) (Photo H) [H&E x16]. The focal fibrosis was detected by MT stain as recorded in Photo I [MT) x16]. Rats treated with HEM before and after BLM showed normal appearance (Photo J) with mild congestion was noticed in the blood vessels (v) (Photos K) [H&E x 16] with no fibrosis in sections stained by MT (Photo L) [MT) x16].

DISCUSSION

A marked inflammation and subsequent repair in the alveoli that lead to remodeling fibrotic changes in the matrix consistently occurs after administration of bleomycin (Thrall and Scalise, 1995). BLM-induced lung injury evolves in two phases in mice. Inflammation and leukocyte cell infiltration in the airways and parenchyma airways is the main features of the first inflammatory phase. The second fibrotic phase peaked between days 9 and 14 and remained elevated till the end of the

experiment and leads to the development of obvious fibrosis **(**Chaudhary *et al.,* 2006). Lactate dehydrogenase (LDH) is of medical importance as it is found largely in lung and heart tissues. Therefore, LDH used as marker of cell damage or inflammation (lung and pulmonary endothelial cell injury) (Drent *et al.,* 1996). In our study, the increase of LDH in BALF reflects a serious injury in the lung tissues-treated with BLM as compared with control untreated animals.

Epithelial cells, and vascular endothelial cells produce NO in the respiratory tract (de Boer *et al.,* 2001). The formed NO degrades into nitrite (NO2-) and nitrate ($NO3^-$). A potent cytotoxic molecule, peroxynitrite (ONOO-) is produced after reaction of NO with superoxide anion. Peroxynitrite causes airway hyper-reactivity and increases leukocytes infiltration (de Boer *et al.,* 2001). In our model the NO level elevated after BLM administration, associated with gross infiltration of inflammatory cells in lung tissues suggesting a link between inflammatory cells and over-production of NO in lung tissues (de Boer *et al.,* 2001). Our results are in agreement with that of Teke *et al*. (2012) who reported similar elevation of NO in BLM-treated rats.

A marked accumulation of inflammatory cells and an increase in the rate of collagen synthesis and deposition is the main characteristic of BLM-induced lung diseases (Verma *et al.,* 2013). The effects of BLM on DNA is postulated to induce the inflammatory and fibroproliferative changes through inflammatory cytokines (IL-6) resulting in abnormal lung collagen deposition (Kalayarasan *et al.,* 2013). This was obvious in our model as BLM administration enhanced the pro-inflammatory cytokine IL-6 and increased hydroxyproline contents a marker of collagen deposition. This could explain the increase in wet lung weight and lung/body weight ratio after BLM as a result of a decline in animals weight and enhancement in collagen deposition that accompanied by fibrotic changes in lung as indicated also from histopathological studies using Masson trichrome stain. BLM also increased MPO levels as an index of neutrophil accumulation a result which is matched with the histopathiological finding of gross inflammatory cell infiltration in lung tissue treated with BLM (Kilic *et al.,* 2014). The enzyme, which is responsible for degradation of the h-aminoalanine moiety of BLM and prevents its metabolite from binding metals (e.g. iron) is deficient in lung tissue. As a result, the lung is a selective target for BLM. Bleomycin can bind metal ions and DNA at the same time forming a complex, and this complex can produce ROS. Therefore, the lung is always vulnerable for oxidative stress damage from BLM (Gutteridge and Xiao Change, 1981). In our study, administration of BLM significantly reduced GSH levels, which is an important antioxidant and decreased SOD activity that is incorporated in detoxification of free radicals. On the other hand, BLM administration increased the MDA levels which is an indicator of lipid peroxidation.

In the lung, heme oxygenase-1 (HO-1) is induced by free radicals overproduction and inflammatory reaction. Hemin (HEM) is HO-1 inducer. In this study, the immunohistochemical analysis and western blot technique of lung tissues revealed strong expression of HO-1 in groups treated with HEM. This cytoprotective enzyme has essential role in protection against toxicants-induced organ damage. In this study, the elevated level of LDH was reduced after using hemin indicating that the lung tissue was protected by HEM against the insulating effects of BLM. This result is consistent with that of Xu *et al.,* (2007) who documented that the leakage of LDH and CK in the coronary effluent was significantly declined in hemin-treated rat hearts. Moreover, HEM reduced the NO levels which were elevated by BLM. NO molecules in the BAL fluid my considered as a free radicals. Ibrahim *et al.* (2014**)** reported that HEM decreases the NO level that was enhanced after induction of ulceration in gastric tissues as a protective action. These results are further supported by the histopathological analysis that reveals alleviation in BLM-induced-lung tissues injury in the group pretreated with HEM.

Heme oxygenase-1 is an enzyme presents in the micro some and plays an important role in suppressing inflammation. The inflammatory responses mediated by macrophage is markedly suppressed after activation of HO-1 (Lee and Chau, 2002). Tu *et al*. (2014) found that hemin, the HO-1 inducer, reduced the activation of the inflammatory signaling molecules JNK and NF-κB, while the HO-1 inhibitor ZnPP abrogated the hemin-induced suppression of inflammatory signaling molecules illustrating that hemin reduces the release of inflammatory cytokine through its inhibitory effect on inflammatory signaling. Also, the phosphorylation of JNK and NF-κB activation is suppressed by the CO producer (CORM-2). Consequently, if we know that CO, a by-product of heme catabolism (by HO-1), has a powerful anti-inflammatory effects through inhibiting JNK and/or NF-κB binding, thus the suppression response of hemin on the activation of the inflammatory signaling molecules may be, at least in part, associated with CO production by HO-1, leading to reduced inflammatory cytokines like IL-6 (Tu *et al.,* 2014). This was obvious in our model, as utilizing HEM significantly produced HO-1 over-expression and in the same time reduced the elevated IL-6 levels and NF-κB over-expression that was augmented by BLM . Yang *et al*. (2012) reported that the induction of HO-1 (by HEM) could reduce all the biochemical indicators of fibrosis (e.g. hydroxyproline) and attenuate the degree of fibrosis caused pathologically by BLM. Our results revealed similar finding of a reduction of hydroxyproline level and amelioration of the histopathological lesions after HEM administration. The

effect of HEM on lung weight was not significant in this study as HEM effect on the fibrotic phase requires more time (21-28 days) to be obvious. Moreover, HEM also reduced the MPO contents in lung tissues-treated with BLM which is in agreement with that of Yoriki *et al*. (2013) who showed that HEM reduced the activity of MPO as an index of neutrophil accumulation in indomethacin-induced small intestinal injury in mice.

Strategies aimed at reducing oxidative stress have been successful in decreasing BLM-induced lung injury and fibrosis. In this study, HEM improved the oxidants status of the lung tissue that was deteriorated after BLM administration. Reduced GSH level and SOD activity are increased after the use of HEM. On the other hand, the elevated MDA (indicator of lipid peroxidation) is reduced after HEM. Al-Kahtani *et al.* (2014) demonstrated that HEM significantly augments GSH level and SOD activity and inhibits the increase of MDA contents in the aorta of T2D rats (Wang *et al.,* 2014**).** The antioxidant and free radicals effects of HEM could be explained on the bases that HO-1, the cytoprotective enzyme blocks injury pathways by catalyzing the division of prooxidant heme into biliverdin (BV), carbon monoxide (CO), and ferrous iron (Fe^{+2})/ferritin. BV formed in this reaction is rapidly recycled by the action of biliverdin reductase into bilirubin which has a higher free radical-scavenging activity and antiapoptotic properties (Fredenburgh *et al.,* 2007). CO has numerous biological functions, including anti-inflammatory properties and also triggers the nuclear factor-erythroid 2-related factor 2 (Nrf2) to increase the expression and function of a battery of antioxidant enzymes (Raval and Lee, 2010).

While some reports suggested that inhibition of HO-1 attenuated pulmonary fibrosis produced after BLM (Atzori *et al.,* 2004). On the other hand, it was demonstrated that adenoviral HO-1 over-expression is beneficial in mice model of bleomycin-induced pulmonary fibrosis (Tsuburai *et al.,* 2002). Similarly, both CO and bilirubin (by-products of heme catabolism by HO-1) have recently been shown to ameliorate fibrosis in the bleomycin model **(**Nagao *et al.,* 2014**;** Wang *et al.,* 2002**).** The results of this study provide further evidence that support a protective role for HO-1 **induction** against pulmonary fibrosis induced by BLM in rats as indicated biochemically and histopathologically.

CONCLUSIONS

The toxicity of BLM on rat lungs is mediated through oxidative stress mechanisms that implicate ROS generation and NO overproduction. Moreover, increased collagen deposition (HP index), MPO, IL-6 production reveals the role of inflammatory mediators and collagen deposition in the pathogenesis of lung fibrosis induced by BLM. The use of HEM with BLM reversed the harmful outcomes induced by BLM through induction of HO-1 enzyme that has multi-pathways including antioxidant mechanism (replenishing of GSH contents, SOD activity and reduction in MDA), anti-inflammatory mechanisms (reduction in MPO, IL-6 and NO lung levels) and anti-fibrotic effects (decline in HP lung contents). This amelioration in biochemical parameters was accompanied with alleviation in hisopathological lesions induced by BLM.

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