Alterations in Thiol/disulphide Homeostasis of Acute Lung Toxicity of Bleomycin in Rats

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ABSTRACT

Bleomycin (BLM)-induced pulmonary fibrosis, a progressive and lethal form of interstitial lung disease, is mediated through the generation of reactive oxygen species. Since the dynamic thiol/disulphide homeostasis is a substantial marker for oxidative stress, for future glutathione based methodological researches on the progressive BLM toxicity in lungs, it is very valuable to show that the toxic mechanism.

We investigated the dynamic thiol/disulphide homeostasis in a rat model with BLM-induced pulmonary fibrosis. BLM (5 mg/kg) or saline was injected into the lungs as 0,1 ml by insuline syringe that inserted into gently opened trachea of anesthetized rats.

Native thiol (SH), total thiol (total SH), and disulphide (SS) levels were measured in rat serum at pre-treatment, 24th hour, 2nd, 4th, 7th, 14th, and 28th days of BLM treatment, with a novel automated method recently described.

The levels of native SH and total SH in BLM group were decreased at 24th hour and this reduction were gradually continued up to 28th day when compared with pre-treatment BLM and/or the saline control group. On the other hand there were significantly a high ratio in BLM group with %SS/SH at 4th day and %SS/total SH at 4th and 28th days while there was no difference at 7th and 14th days.

Results clearly indicate that the thiol/disulphide homeostasis can be acutely disturbed in the experimental model of BLM-induced fibrosis. So this data offer a new modeling option to discuss the therapy strategies and the effect mechanisms of BLM-induced lung toxicity.

Introduction

Idiopathic pulmonary fibrosis is a progressive lung disease along with respiratory failure. Bleomycin (BLM) is used as a tool to understand the key mechanisms of lung fibrosis. It is a very well known antineoplastic agent via an oxygen-dependent mechanism of DNA cleavage, causes progress if interstitial pulmonary fibrosis [1]. Just before the 1970s, researches had discriminated that DNA could be bind and scission by it [2]. A ferrous iron-bleomycin complex reacts with oxygen and is oxidized to the ferric state. Glutathione (GSH) is able to bind to this ferric iron-bleomycin complex and reduce to the active ferrous-iron complex. So a catalytic oxidation-reduction cycle in which reducing equivalents from GSH are ultimately transferred to oxygen resulting in the formation of reactive oxygen species (ROS) [3–5]. In fact the BLM action on the DNA of living cells is modulated by thiol-containing compounds [6]. Several reports point out that the pathogenesis of BLM-induced fibrosis is mediated through the generation of ROS which cause the peroxidation of membrane lipids and DNA damage [7,8]. Organic thiol compounds contain a sulfhydryl group (–SH) composed of a sulphur and a hydrogen attached to a carbon atom. The tissue thiols are formed by albumin thiols, protein thiols and low molecular weight thiols that include cysteine (Cys), cysteinylglycine, glutathione, homocysteine and

γ-glutamylcysteine. Thiol compounds can undergo oxidation reaction via oxidants and form disulphide (RSSR) bonds. Under oxidative stress conditions, the oxidation of Cys residues can lead to the reversible formation of mixed disulphides between protein thiol groups and low-molecular-mass thiols [4-6]. The formed disulphide bonds again reduced to thiol groups; thus, dynamic thiol–disulphide homeostasis is maintained [9]. Moreover, dynamic thiol disulphide homeostasis is being increasingly implicated in many disorders. There is a growing body of evidence that an abnormal thiol disulphide homeostasis state is involved in the pathogenesis of a variety of events, cardiovascular disease [10,11], cancer [12,13], rheumatoid arthritis [14], and psoriasis [15]. The gauging of dynamic thiol disulphide homeostasis is revealed valuable notice on normal physiologic or abnormal pathogenic conditions. Recent clinical studies have shown that the dynamic thiol disulphide homeostasis is disturbed in the respiratory abnormalities and lung diseases such as apnea, COPD and in healthy or unhealthy smokers and workers exposed to several particules or gases [16-20]. Therefore the dynamic thiol disulphide homeostasis status can play critical roles in antioxidant protection, detoxification, signal transduction, apoptosis, regulation of enzymatic activity and transcription factors and cellular signalling mechanisms in the BLM-induced fibrosis.

This research was designed to show that the dynamic thiol/disulphide homeostasis as a biomarker for oxidative stress in the future methodological models on the progressive BLM toxicity in lungs. Hence it may be very valuable to clear the toxic mechanisms of lung fibrosis.

Experimental Section
Animals and experimental procedure
Female Wistar-albino rats were housed in room at $22 \pm 2°C$ with 40% relative humidity and with a 12-hr light ± dark cycle. They were fed with a standard rat chow and tap water ad libitum. The studies were conducted in accordance with the standards and permission established by The Ethics Committee of Animal, The University of Sutcu Imam.

The respiratory pathogen-free rats, weighting 180-220 g, were divided into a saline control group (SA) and a group (BLM) receiving bleomycin sulphate (Bleocin, Nippon Kayaku Co., Tokyo, Japan) dissolved in sterile physiological saline. The studies were carried out in two series as periods of 7 or 28 days (A or B). Single doses of BLM (5 mg/kg) or saline was injected into the lungs as 0,1 ml by insuline syringe that inserted into gently opened trachea of anesthetized rats (n=6-9). Animals were observed day by day and their body weights were recorded. Lung tissue was weighted to determine the lung index. Lung index was determined by dividing lung weight (g) by body weight (g) and multiplying by 100. As some of them were dead during the experiment procedures and period, they were took out from the group of that time.

Measurement of Thiol Biomarkers
The volumes of 0,3-0,4 ml for blood samples were obtained from their tail veins of BLM and saline control animals were collected at pre-treatment, and 24th hour, 2nd, 4th, 7th, 14th, and 28th days after treatments, transferred into tubes to measure of levels of native thiol (SH), total thiol (total SH), and disulphide (SS) biomarkers of pulmonary fibrosis. Sera was separated by centrifugation at 1500 g for 10 minutes and stored at -80°C until analysis.

We used a novel automated method recently described and developed by Erel and Neselioglu [9] to asses serum dynamic disulphide/thiol homeostasis as SH, total SH, and SS, and then calculated the %SH/total SH, %SS/SH, and %SS/total SH ratios. In short, dynamic disulphide bonds (-S-S-) in the serum were reduced to form free thiols (-SH) by NaBH4. Formaldehyde was used to consume and suspend the remaining reductant NaBH4 entirely, and all SH groups, including the reduced and native groups, were evaluated after reaction with 5,50-dithiobis-(2-nitrobenzoic) acid. Half of the difference between the total SH and native SH levels was due to the dynamic SS level. After measuring the levels of native SH and SS, the SS/SH ratio was calculated.

Hydroxyproline Assay
At the 7th or 28th day after the onset of treatment, rats were anaesthetized with sodium thiopental (70 mg/kg, i.p.) and lung lobes were excised, washed in ice-cold saline, and then quickly frozen in liquid nitrogen. The frozen lung lobes of each animal were thawed and homogenized in isotonic saline with a Polytron. The homogenates were transferred to tubes and stored at -80°C until assayed. The lung hydroxyproline content, index of fibrosis, was measured by the technique described by Woessner [21].

GSH Determination
Lung tissues were homogenized in 10 ml TCA (trichloroacetic acid) which is at the rate of 10%, and then centrifuged at +4 °C for 15 minutes. Afterwards, 0,5 ml of supernatant was taken, and mixed with 0,3 M 2 ml Na2HPO4. The mixture was thoroughly vortexed. This mixture was vortexed by the addition of 0,2 ml DTBN (Dithiobisnitrobenzene: prepared by dissolving in 1% sodium citrate). Finally it's absorbance was measured at 412 nm.

Measurement of MDA
Lung tissues were homogenized in 10 ml TCA (trichloroacetic acid) which is at the rate of 10%, and then centrifuged at +4°C for 15 minutes. 750 µl of the supernatant which was obtained was mixed with 0.67% TBA (thiobarbituric acid) in a ratio of 1:1. Afterwards, the solution was left in the water bath for 15 minutes. Finally, the absorbance was measured spectrophotometrically at 535 nm.

Statistical analyses
Data are expressed as the mean ± S.D of n experiments. Analysis of variance followed by post hoc tests or nonparametric test was applied as appropriate. Significance was assumed for $p<0.05$.

Results and Discussion
Body Weight and Lung Index Results
Treatment with BLM resulted in marked decrease in body weight compared to saline control group ($p<0.05$). As can be seen Figure 1A, body weight of the saline control group animals increased

in parallel with the day's increase, but BLM group animals's decreased inversely proportional with these results. On the other side, treatment of rats with BLM showed an increase in lung index, a parameter for lung edema, compared with that of the saline control group at both 7th and 28th days, ($p<0.05$) (Figure 1B).
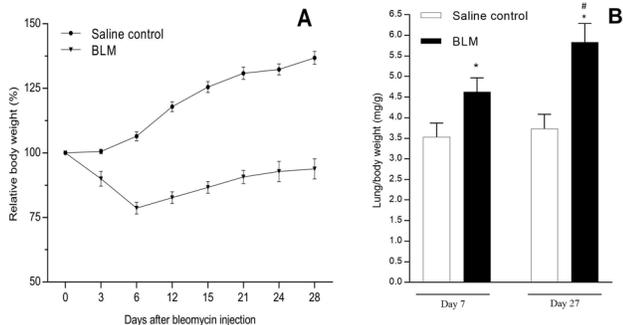

Figure 1: (A) Body weight loss in bleomycin treated rats. The body weight on day 0 was defined as 100%. The relative body weight was calculated as a percentage of that measured on day 0. (B) Lung index in the bleomycin treated rats. Rats were sacrificed on days 7 and 28 after bleomycin injection. Lung index was calculated as the ratio of lung wight (g) to body weight (g) of each rat. Values are expressed as the mean ± Standard deviation (n=6). *Values significantly different from saline controls on days both 7 and 28. #Values significantly different from BLM group on day 7. $p<0.05$.

Hydroxyproline Results

In order to evaluate the extent of fibrosis, the collagen deposition in lung tissues was determined by measuring the hydroxyproline content. As shown in Figure 2A, at day 28, the lung hydroxyproline level in the BLM-treated rats was increased markedly compared with that of the saline control rats ($p<0.05$).
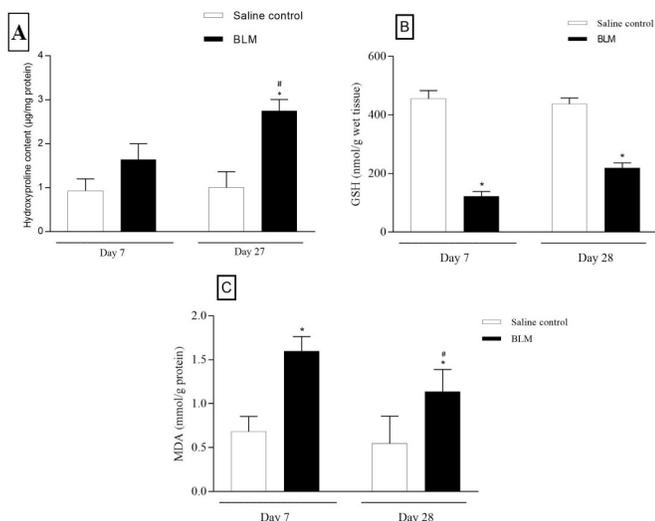

Figure 2: (A) Lung hydroxyproline levels in bleomycin treated rats. (B) Lung GSH levels in bleomycin treated rats. (C) Lung MDA levels in bleomycin treated rats. Rats were sacrificed on days 7 and 28 after bleomycin injection. Values are expressed as the mean ± Standard deviation (n=6). *Values significantly different from saline controls on days both 7 and 28. #Values significantly different from BLM group on day 7. $p<0.05$.

When the 7-day serie and the 28-day serie were compared, the lung hydroxyproline level in the BLM-treated rats was significantly higher at day 28 than at day 7 ($p<0.05$).

GSH levels in lung tissue
GSH were measured in lung tissue at 7th and 28th days after BLM treatment. A significant decrease in GSH was observed in BLM-treated rats ($p<0.05$) (Figure 2B).

MDA levels in lung tissue
The MDA levels were measured in lung tissue at 7th and 28th days after BLM treatment. The MDA levels in the BLM-treated rats were increased markedly compared with that of the saline control rats in the both of series. When the 7-day serie and the 28-day serie were compared, the lung MDA levels in the BLM-treated rats were significantly lower at day 28 than at day 7 ($p<0.05$) (Figure 2C).

Thiol Results
The levels of native and total SH in BLM group were significantly decreased at 24th hour and this reduction was gradually continued up to 28th day when compared with pre-treatment BLM and/or the saline control group ($p<0.05$).
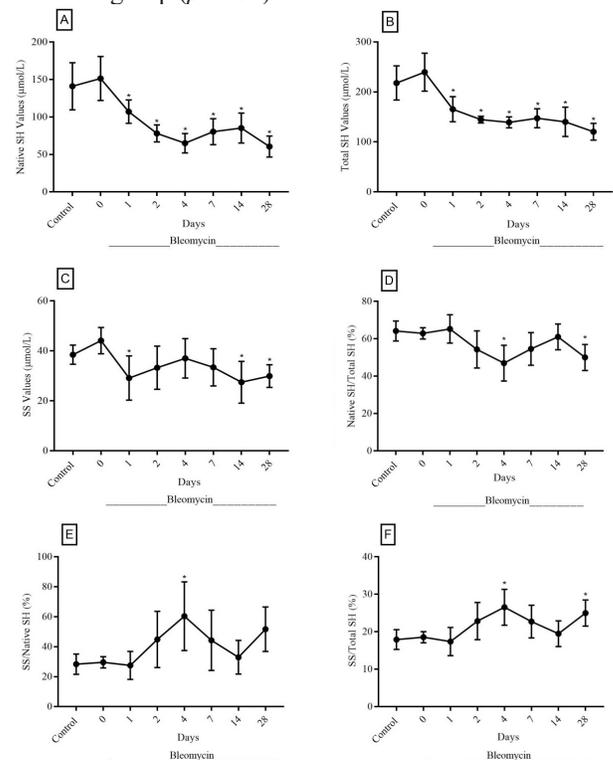

Figure 3: Time-course changes in thiol levels of serum of bleomycin treated rats. (A) Native thiol (SH) levels. (B) Total thiol (total SH). (C) Disulphide (SS) levels. (D) %SH/total SH ratios. (E) %SS/SH ratios (F) %SS/total SH ratios. Values are expressed as the mean ± Standard deviation (n=6). *Values significantly different from saline controls and/or on day 0 (preadministared BLM). $p<0.05$.

On the other hand there were significantly high ratios in BLM group with %SS/SH at 4th day, and %SS/total SH at 4th and 28th days, while there were no differences in the thiol ratios at 7th and

14th days when compared with pre-treatment BLM and/or saline control groups ($p<0.05$) (Figure 3A-F).

Bleomycin fibrosis is a progressive and constant disease or advers event that unfortunately there is no effective treatment. Bleomycin induced animal lung injury has been widely used as a model of human lung fibrosis. We were used a single intratracheal dose of BLM as a tool to get experimental lung fibrosis in rats as well earlier studies [8,22]. Bleomycin treatment resulted in body weight loss, lung index increase, progressive fibrosis characterised with elevation of hydroxyproline content in rat lung tissues. First of all, this study is a new methodologic approach on the patho-toxicologic mechanism of bleomycin-induced lung fibrosis. The dynamic thiol–disulphide homeostasis was studied with serum thiol biomarkers such as SH, total SH, and SS in animal lung fibrosis. The levels of SH and total SH in BLM group were decreased at 24th hour and this reduction was gradually continued up to 28th day when compared with pre-treatment BLM and/or the saline control group. On the other hand there were significantly high ratios in BLM group with %SS/SH at 4th day and %SS/total SH at 4th and 28 th days while there were no difference at 7th and 14th days.

Firstly, in order to evaluate the extent of fibrosis, the collagen deposition in lung tissues was determined by measuring the hydroxyproline content. At 28th day after bleomycin, the lung hydroxyproline level in the bleomycin-treated rats was increased markedly compared with that of the saline control rats. When the 7-day serie and the 28-day serie were compared, the lung hydroxyproline level in the BLM-treated rats was significantly higher at day 28 than at day 7 ($p<0.05$). So fibrosis development is very evident at 28th day after BLM treatment to rats. It is maybe period of irreversible pathophysiological processing of fibrosis [7,22].

Secondly, in order to evaluate the lipid peroxidation in lung tissues was determined by measuring the MDA level. Although an increase was detected in MDA levels both in 7-day and 28-day series with BLM, the increase in MDA levels in the 7-day serie was higher than that of 28-day. The inflammatory response observed following bleomycin treatment include oxidative stres is one of the main mechanisms in the development of fibrosis which a complex sequence of reactions. BLM is able to mediate DNA strand scission in presence of iron and oxygen, producing single- or double-strand breaks with consequent higher ROS [7]. Lung tissue has defence mechanisms that include non-enzymatic and antioxidant enzymes associate with ROS in pathogenesis of pulmonary injury. The balance in between ROS generation and antioxidant systems can shift to high ROS production and weaken antioxidant defences in cell injury. In BLM-induced pulmonary injury can be seen high oxidative stress along with increased MDA level of lipid peroxidation [7]. The results of this study showed that MDA level is increased in the lung tissue after a single intratracheal BLM injection to rats.

Thirdly, in order to evaluate the antioxidant defence in lung tissues GSH levels were measured. It was observed decreased GSH levels

in BLM-induced lung fibrosis. High oxidative stress is associated with the decreased GSH content in tissues [7]. BLM is to exert the cytotoxic effect by cleaving DNA in a process dependent on the presence of both molecular oxygen and a metal ion. BLM binds to iron and this complex cause to cleave DNA in the presence of hydrogen peroxide. Both Fe(II) and O2 work as co-factors in DNA cleavage. Also BLM binds DNA and Fe(II) and the presence of molecular oxygen leads to the release of hydroxyl radicals. The free radicals can cause DNA breaks that ultimately lead to cell death [5,8,22]. Thiol molecules in tissues or blood, the sulfhydryl group (–SH), protects against inflammation associated with oxidative stress. Blood thiol reserve is basically constituted by protein thiols, and by low molecular weight thiols including cysteine, cysteinylglycine, glutathione, homocysteine, and c glutamylcysteine. Serum native thiol groups can be oxidized to SS groups. The levels of SH groups in blood and tissue can be depleted by oxidation, although it is possible for newly formed SS groups to be converted back into SH groups, thereby sustaining SH/SS homeostasis [9].

In fact, the main purposes of this research is: 1) to prove the dynamic thiol/disulphide homeostasis, and 2) to understand whether there is critical role of it in period or processes of fibrosis development. From this perspective, this manuscript is to present a very new methodological approachment. According to the results of this research, inflamatuar processes caused by BLM is to starting acutely within first 24 hour after BLM treatment. For this reason, from now on, the researchers need to take into account the unbalance state or disruption of thiol/disulphide homeostasis in the BLM-induced lung fibrosis models will be planned in animals. According to our opinion, the inflammatory processes begin right (JUST?) after BLM treatment in lung. The one of first signs of lung inflammatory in fibrosis may be disruption of dynamic thiol/disulphide homeostasis in serum [23].

To date it has been declared in a number of experimental and clinical studies that the biomarkers of oxidative stress is very marked in pathogenesis of lung fibrosis [7,22]. The results of this research revealed that the thiol/disulphide homeostasis is disturbed acutely after BLM treatment and interestingly fully failured in the long processes of lung fibrosis. The disulphide levels or the % ratio of SS/total SH in the beginning of toxicity may be a biomarker of oxidative stress in lung fibrosis. In fact, in the evaluation of lung fibrosis, this study will enable both experimental researchers and clinicians to determine the oxidative status of animals or patients with a practical and fully automated method. Since thiol/disulphide homeostasis is disturbed in lung fibrosis, the further researches are recommended for good therapeutic applications with the thiol agents and antioxidants.

In this research, it is obtained the higher serum disulphide levels of BLM-induced pulmonary fibrosis in rats than saline controls. The high disulphide levels measured in fibrosis can be a result of oxidative stress induced by BLM [23,24]. The use of thiol contain agents can be valuable in the severe lung fibrosis in chronic step. In addition, the disulphide to native thiol and disulphide to total

thiol % ratios were higher in BLM-induced fibrotic rats than saline control. The redox state of the thiol/disulphide couple is to sign as an indicator of oxidative stress [24]. GSH is the main intracellular thiol pool and is the basic endogenous antioxidant in cells. It is used in enzymatic reactions and to take out peroxides and in non-enzymatic reactions to maintain the antioxidants [7]. The GSH-related detoxification of ROS is achieved through two general mechanisms: i) reacting with ROS; ii) inactivation of ROS catalyzed by GSH peroxidase. GSH disulphide, oxidized GSH, is produced due to these reactions [4,23,25]. Thiols which is the functional group of GSH can undergo a wide range of modifications, which involve reversible oxidation of the thiol to disulphide, including intra- and intermolecular disulphides between polypeptides and GSH-polypeptide. The reversibility of these oxidation reactions allows thiol groups to act as versatile transducing elements in a variety of low molecular weight mass metabolites and proteins. Thiol groups are oxidized to disulphides in oxidative stress, so thiol/disulphide ratio offers a view of cellular oxidative stress [25].

Recently studies have shown that the thiol/disulphide homeostasis is disturbed in lung diseases such as obstructive sleep apnea syndrome [17], COPD [26], asthma [26], silicasis [19], infectious pneumonia [27], pulmonary thromboembolism [28]. Dinc et al. [16] declared that the SH level and %SH/total thiol ratio are low in patients with obstructive sleep apnea syndrome. The decreased thiol levels are indicative of the inadequacy of the antioxidant defense in pregnant women with obstructive sleep apnea syndrome [17]. Plasma total thiol levels are reduced in patients with COPD or asthma. Furthermore the treatment with antioxidants is able to restore plasma total thiol levels in these pulmonary diseases [29]. As well known, smoking can cause a clear oxidative stress. Solak et al. [30] revealed that thiol/disulphide homeostasis shifts through disulphide side in oxidative stress of smokers. Similar findings have been obtained in silica-exposed workers, along with high serum disulphide level which is biomarker of oxidative stress [19].

Without doubt, the most common route of BLM treatment is intratracheal, causing an inflammatory response with several steps [19,31,32]. To our knowledge, this is the first published study to determine the time course and detection of the dynamic thiol/disulphide levels in development of inflammation in BLM-induced lung fibrosis. According to the results of this study the BLM caused pulmonary inflammation events is right starting at the first day associated with oxidation-peroxidation of cells and defense mechanisms of thiol redox. At the 4th day, the dynamic thiol/disulphide homeosthasis is shifted to disulphide side. Then after the dynamic thiol/disulphide levels are to show fluctuations in serum. That event it may be associated with the defensive efforts of pulmonary cells to return the physiologic conditions in next inflammatory steps. Even so fibrosis is detected at 7 day, fibrotic step is persisting at the 28th day with high hydroxyproline levels in lung tissue.

Conclusion

Results clearly indicate that the thiol/disulphide homeostasis can be acutely disturbed in the experimental model of BLM-induced fibrosis. So these datas offer a new modeling option to discuss the therapy strategies and the effect mechanisms of BLM-induced lung toxicity.

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