

Protective Effect Of Thymoquinone And Aminoguanidine Against Bleomycin Induced Lung Damage: Possible Role Of Nitric Oxide Synthase

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Citation

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Abstract

Bleomycin-induced lung injury is due to oxidative damage produced by free radicals generations. The present study was undertaken to investigate whether aminoguanidine, an inhibitor of Nitric Oxide (NO) synthase, and thymoquinone, a potent superoxide radical scavenger, can protect against bleomycin-induced lung damage in rats. Administration of bleomycin, 15 mg/kg, i.p. every other day for 4 weeks, induced lung toxicity as indicated by a significant increase in the level of lipid peroxide, significant depletion of the reduced glutathione in lung tissue and significant increase in the activity of antioxidant enzymes glutathione peroxidase and glutathione S-transferases. Oral administration of aminoguanidine (50 mg/100mL) or thymoquinone (5 mg/100mL) for one day before and during the period of bleomycin treatment leads to normalize the alterations in the biochemical parameters induced by bleomycin toxicity. In conclusion, this study demonstrated that both thymoquinone and aminoguanidine significantly attenuate the development of pulmonary toxicity. The thymoquinone is a potent superoxide radical scavenger, scavenging power being as effective as superoxide dismutase against superoxide. The superoxide scavenging and anti-lipid peroxidation may play a part in the protective effect of thymoquinone against bleomycin-induced lung toxicity. However the effect of aminoguanidine was predominantly mediated by inhibition of inducible nitric oxide synthase (iNOS) activity, thereby reducing peroxynitrite formation. We propose that the development of a more specific and potent inhibitors of iNOS might be beneficial in the prevention and treatment of lung toxicity.

INTRODUCTION

Bleomycin, a glycopeptide antibiotic, is useful against a broad spectrum of human cancers such as squamous cell carcinomas, lymphomas and testicular tumors (Suntres and Shek, 1997, Won et al.,2012). Bleomycin therapy is, however, usually associated with the development of dose- and time-dependent life-threatening pneumonitis that can progress to interstitial pulmonary fibrosis with features similar to those observed in idiopathic pulmonary fibrosis (Chandler, 1990; Smith et al., 1996; Keane et al., 1999, Erdogan et al.,2006). Induction of lung injury by bleomycin has been observed in several animal species and therefore, animal models of bleomycin-induced lung injury are used as representative models for the study of human pulmonary fibrosis (Ortize et al., 1998). The pathogenesis of this toxicity seems to be multifactorial and indeed several mechanisms have been proposed to be implicated in the induction of pulmonary toxicity following bleomycin administration. Among these mechanisms are elevation of some cytokines such as tumor necrosis factor (TNF)- α (Ortize et al.,1998,

Liang et al.,2011), stimulation of endothelial cell transforming growth factor (TGF)- β_1 (Khalil et al.,1998), depletion of NAD and ATP (O'Neil, and Giri,1994), involvement of platelet activating factor (PAF) and generation of reactive oxygen species (ROS) such as superoxide anion and hydroxyl radicals (Habib et al.,1993; Karam et al.,1998).

Superoxide radical changes to hydrogen peroxide by superoxide dismutase enzyme, which in turn changes into hydroxyl radicals or detoxifies by catalase or glutathione peroxidase in the presence of glutathione (GSH) to form water and oxidized glutathione (GSSG) (Gragus & klaassen, 1996).

These reactive oxygen species especially hydroxyl radicals react with polyunsaturated fatty acids to yield lipid hydroperoxide. These initiate a lipid radical chain reaction, which can cause oxidative damage to the cell, leading to increase membrane fluidity, permeability, loss of membrane integrity, dysfunction of mitochondria and sarcoplasmic

reticulum and altered calcium homeostasis. The production of oxygen free radicals from bleomycin toxicity leads to arrhythmia and myocardial necrosis. (Ramos , et al.,1996).

Thymoquinone is a potent superoxide radical scavenger, its scavenging power being as effective as superoxide dismutase against superoxide. The superoxide scavenging and anti-lipid peroxidation may play a part in the protective effect of thymoquinone against doxorubicin -induced cardiotoxicity (Nagi and Mansour ,2000).

Nitric oxide (NO) is a small membrane-permeable gas that acts as a mediator of many physiological functions, including vascular relaxation via cyclic guanine monophosphate (cGMP) accumulation (Moncada, et al., 1991). It has been reported that NO can rapidly combine with superoxide to form peroxynitrite which result in nitric oxide scavenging (Pryor and Squadrito, 1995). Also, superoxide can trap and hence modulate the effect of nitric oxide, by controlling superoxide dismutase levels, therefore can influence the reaction pathways open to nitric oxide. Peroxynitrite is a potent and versatile oxidant that can attack a wide range of biological targets (Pryor and Squadrito, 1995).

Aminoguanidine, a compound structurally similar to L-arginine (the substrate for nitric oxide), inhibits nitric oxide formation and interferes with the formation of peroxynitrite (Abraham et al., 2009) . As Bleomycin induced pulmonary cell damage is attributed to free radical formation, a possible protection could be achieved by inhibiting NO synthase. The present study was undertaken to investigate whether inhibition of nitric oxide synthase by aminoguanidine, an inhibitor of NO synthase, can protect against bleomycin-induced lung toxicity and to investigate whether thymoquinone, a potent superoxide radical scavenger can protect against bleomycin -induced lung toxicity.

MATERIALS AND METHODS

DRUGS AND CHEMICALS

Bleomycin hydrochloride (BL) was supplied as bleomycin ampoules (15 mg) from Nippon Kayaku Co., LTD. (Tokyo, Japan). Aminoguanidine hydrochloride (AG) were obtained from Sigma (St. Louis, MO,USA).While thymoquinone (THQ) was obtained from Aldrich Chemical Co.. Thiobarbituric acid (TBA) was a product of Fluka (Buchs, Switzerland). All the other chemicals were of the highest analytical grade and obtained from commercial suppliers.

LABORATORY ANIMALS

Male Swiss albino rats were used in this study. They were obtained from the Experimental Animal Care Center of Faculty of Science in Tanta University. The average body weight of animals was 200 g. and they were maintained under similar housing conditions with free access to food (Purina Chow) and water. The protocol of this study has been approved by Research Ethics Committee of College of Medicine, Tanta University, Tanta, Egypt.

EXPERIMENTAL PROTOCOL

Rats were randomized into six groups, each consisted of five animals. The first group (BL group) received i.p. injection of BL at a dose of 15 mg/kg every other day for four consecutive weeks (Daba et al., 2002), while the second group, the aminoguanidine group (AG group), received aminoguanidine 50 mg /100ml in drinking water (Cartledge et al., 2001) . The third group, the thymoquinone (THQ) group, was given thymoquinone 5 mg /100ml in drinking water (Badary et al .,1997). The fourth group (AG – BL) received aminoguanidine in drinking water and one day later start BL treatment as in the first group. The fifth group (THQ – BL group) received thymoquinone in drinking water, and one day later the rats treated with BL as in the first group. The sixth group, the control group, received an i.p. injection of normal saline every other day for four consecutive weeks. Aminoguanidine and thymoquinone in the respective groups were given in the drinking water for the whole period (4 weeks) of experiment.

Twenty-four hours after the last dose of the specific treatment, all rats were weighed and then sacrificed by cervical dislocation.

PREPARATION OF LUNG HOMOGENATES FOR BIOCHEMICAL MEASUREMENTS

The isolated lungs were rinsed in chilled 1.15 % KCl (pH 7.4) and weighed quickly. Lung / body weight ratio was then determined. Homogenization was carried out in ice-cold KCl (1.15%, pH 7.4) to yield a 10% (w/v) tissue homogenates.

DETERMINATION OF REDUCED GLUTATHIONE AND LIPID PEROXIDATION IN LUNG TISSUES

The tissue levels of the acid soluble thiols, mainly GSH, were assayed spectrophotometrically at 412 nm, according to the method of Ellman,(Ellman,1959) using spectrophotometer. The contents of GSH were expressed as mmol/g wet tissue. The degree of lipid peroxidation in lung

tissues was determined by measuring thiobarbituric acid reactive substances (TBARS) in the supernatant tissue from homogenate (Ohkawa et al.,1979). The homogenates were centrifuged at 1,500 g and supernatant was collected and used for the estimation of TBARS. The absorbance was measured spectrophotometrically at 532 nm and the concentrations were expressed as nmol TBARS/g wet tissue.

DETERMINATION OF GLUTATHIONE S TRANSFERASE, GLUTATHIONE PEROXIDASE AND ALKALINE PHOSPHATASE ACTIVITY IN LUNG TISSUES

Glutathione-S-transferase (GST) activity in lung homogenates was determined spectrophotometrically at 30°C with 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate (Habig et al., 1974). The reaction was monitored at 340 nm and the activity of GST is expressed as $\mu\text{mol GSH-CDNB conjugates formed per min per g tissue}$.

Glutathione peroxidase (GSH-PX) activity was estimated in tissue homogenates by a kinetic assay at 37°C using a test reagent kit for Se-GSH-Px (RANSEL, Randox, UK). Absorbance was measured at 340 nm and the results are expressed as $\mu\text{mol/min /g tissue}$ (Paglia and Valentine,1967).

Alkaline phosphatase (ALK) activity was determined in lung tissue colourimetrically at 510 nm (Belfield, and Goldberg, 1971) using a test reagent kit (BioMerieux, France). The results are expressed as $\mu\text{mol/min /g tissue}$.

STATISTICAL ANALYSIS

Data are expressed as means \pm S.E.M. Statistical significance was taken as $p < 0.05$, using one-way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparisons test to judge the difference between various groups.

RESULTS

GENERAL OBSERVATION

Administration of BL, at a dose of 15 mg/kg every other day for four consecutive weeks, did not significantly alter body or lung weight of the treated rats. Also, the ratio of lung weight/body weight did not alter after treatment. Similarly, AG or THQ or combined AG or THQ with BL treatment did not alter the body weight, lung weight or the ratio of lung weight / body weight.

EFFECTS OF BLEOMYCIN, AG AND THQ ON

THE MEASURED BIOCHEMICAL PARAMETERS IN THE LUNG HOMOGENATES

The effects of BL, AG, THQ and their combination are demonstrated in Figure 1. The control value of GSH in the lung homogenate of normal rats was $1.48 \pm 0.07 \mu\text{mole/g tissue}$. Administration of BL significantly decreased the GSH level by 54% of control value. However, combined administration of BL and AG or THQ significantly altered the GSH levels (1.44 ± 0.03 , $1.55 \pm 0.14 \mu\text{mole/g tissue}$ respectively) when compared to normal lung homogenate. AG or THQ alone did not significantly alter the level of GSH (Fig 1).

BL treatment significantly increase the levels of LP in the lung homogenate, the LP level in normal homogenate was $152.3 \pm 6.4 \text{ nmole MDA/g tissue}$, while after treatment with BL the LP level increased by 28.2% (Fig 2). Similarly, co-administration of AG or THQ with BL decrease the LP level to reach 139 ± 8.5 and $149 \pm 6.8 \text{ nmole MDA/g tissue}$ respectively (Fig 2).

The GST activity in normal homogenate was $3.5 \pm 0.4 \mu\text{mole/ min /g tissue}$. Treatment with BL, significantly alter the GST activity as can be seen in figure 1b. Similarly, co-administration of AG or THQ with BL decrease the GST activity level to reach 3.5 ± 0.53 and $3.7 \pm 0.61 \text{ nmole MDA/g tissue}$ respectively (Fig 3).

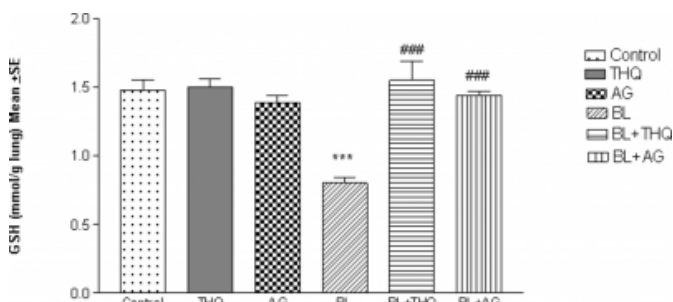
The GSH-Px activity in normal lung homogenate was $18.2 \pm 2.8 \mu\text{mole/ min /g tissue}$. BL treatment significantly increased the activity by 174%. Co administration of AG with BL treatment significantly alters the activity of GSH-px compared to the control value (Figure 4). However, Co administration of THQ with BL, the activity of GSH-px returned back to nearly normal value.

The normal alkaline phosphatase (ALK) activity in the lung homogenate was $10.4 \pm 0.8 \mu\text{mol/min /g tissue}$. Treatment of rats with BL significantly affects the ALK activity to $15.4 \pm 0.8 \mu\text{mol/min /g tissue}$. Co administration of AG with BL treatment did not significantly alter the activity of ALK compared to the control value. However, Co administration of THQ with BL, the activity of ALK returned back to nearly normal value (Figure5).

Protective Effect Of Thymoquinone And Aminoguanidine Against Bleomycin Induced Lung Damage: Possible Role Of Nitric Oxide Synthase

Figure 1

Figure 1: Effect of THQ and AG on Bleomycin-induced changes in the levels of glutathione (G SH) in lung

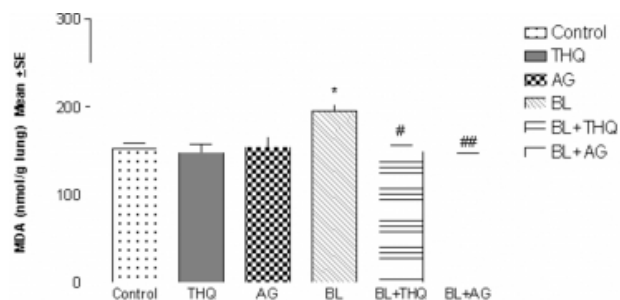


Bleomycin (15 mg/kg, i.p. every otherday for 4 weeks). AG (50 mg/kg, p.o.) were administered for 4 weeks. THQ (5 mg/kg, p.o.) were administered for 4 weeks. In case of combined treatment, AG or THQ was given ond day prior to the administration of bleomycin. Twenty four hours after the last dose of BL, rats were sacrificed and lungs were rapidly excised and homogenized in 1.15 % KCl (pH 7.4) to yield 10% tissue homogenates.

* Significant difference from control group # Significant difference from bleomycin group
P<0.05 ### P<0.01 ##### P<0.001

Figure 2

Figure 2: Effect of THQ and AG on Bleomycin-induced changes in the levels of lipid peroxides (MDA) in lung

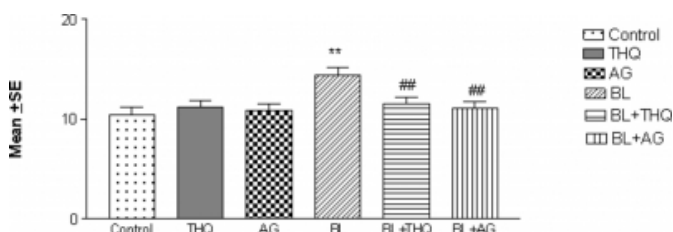


Bleomycin (15 mg/kg, i.p. every otherday for 4 weeks). AG (50 mg/kg, p.o.) were administered for 4 weeks. THQ (5 mg/kg, p.o.) were administered for 4 weeks. In case of combined treatment, AG or THQ was given ond day prior to the administration of bleomycin. Twenty four hours after the last dose of BL, rats were sacrificed and lungs were rapidly excised and homogenized in 1.15 % KCl (pH 7.4) to yield 10% tissue homogenates.

* Significant difference from control group # Significant difference from bleomycin group
P<0.05 ### P<0.01 ##### P<0.001

Figure 3

Figure 3: Effect of THQ and Ag on Bleomycin-induced changes in the levels of the activities of glutathione S-transferases (GST) in lung

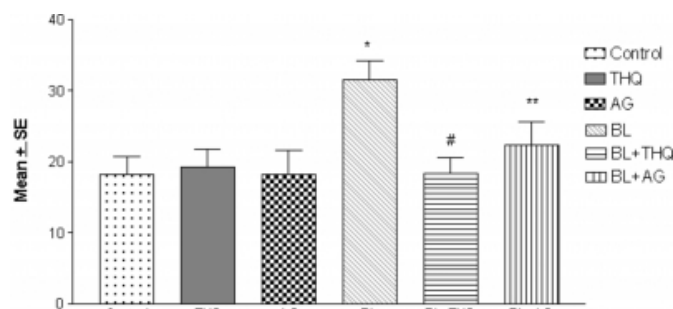


Bleomycin (15 mg/kg, i.p. every otherday for 4 weeks). AG (50 mg/kg, p.o.) were administered for 4 weeks. THQ (5 mg/kg, p.o.) were administered for 4 weeks. In case of combined treatment, AG or THQ was given ond day prior to the administration of bleomycin. Twenty four hours after the last dose of BL, rats were sacrificed and lungs were rapidly excised and homogenized in 1.15 % KCl (pH 7.4) to yield 10% tissue homogenates.

* Significant difference from control group # Significant difference from bleomycin group
P<0.05 ### P<0.01 ##### P<0.001

Figure 4

Figure 4: Effect of THQ and AG on Bleomycin-induced changes in the activities of glutathione peroxidase (G SH-Px) enzyme in lung

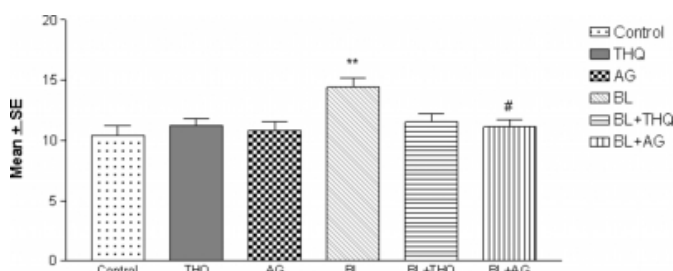


Bleomycin (15 mg/kg, i.p. every otherday for 4 weeks). AG (50 mg/kg, p.o.) were administered for 4 weeks. THQ (5 mg/kg, p.o.) were administered for 4 weeks. In case of combined treatment, AG or THQ was given ond day prior to the administration of bleomycin. Twenty four hours after the last dose of BL, rats were sacrificed and lungs were rapidly excised and homogenized in 1.15 % KCl (pH 7.4) to yield 10% tissue homogenates.

* Significant difference from control group # Significant difference from bleomycin group
P<0.05 ### P<0.01 ##### P<0.001

Figure 5

Figure 5: Effect of THQ and AG on Bleomycin-induced changes in the activities of alkaline phosphatase (ALK) enzyme in lung



Bleomycin (15 mg/kg, i.p. every otherday for 4 weeks). AG (50 mg/kg, p.o.) were administered for 4 weeks. THQ (5 mg/kg, p.o.) were administered for 4 weeks. In case of combined treatment, AG or THQ was given ond day prior to the administration of bleomycin. Twenty four hours after the last dose of BL, rats were sacrificed and lungs were rapidly excised and homogenized in 1.15 % KCl (pH 7.4) to yield 10% tissue homogenates.

* Significant difference from control group # Significant difference from bleomycin group
P<0.05 ### P<0.01 ##### P<0.001

DISCUSSION

Bleomycin, a highly effective antitumor agent, is known to produce lung injury that limits its clinical use (Chandler,1990; Smith et al.,1996; Keane et al.,1999, Erdogan et al.,2006) . This lung injury is characterized by acute pulmonary inflammatory reaction associated with pulmonary edema and possibly pulmonary fibrosis(Ortiz et al.,1998) . Bleomycin has also been reported to mediate pulmonary endothelial cell damage (Orr et al .,1988) . In the present study, bleomycin-induced lung injury was detected by the alterations observed in the levels and activities of the measured biochemical parameters. Treatment of rats with bleomycin significantly increased the lipid peroxide level concomitant with reduction in glutathione level in the lung

homogenates. The primary mechanism by which bleomycin induces pulmonary toxicity is thought to be related to redox cycling of an iron-bleomycin complex that catalyzes the formation of superoxide and hydroxyl radicals and causes DNA strand scission and lipid peroxidation (Muraoka et al., 1986, Kara et al.,2010) .

The observed increase in LP content of lung tissue is in accordance with the finding of Kara et al.,2010. Also, Kalayarasan et al., 2008 who found a significant increase in the amount of thiobarbituric acid reacting products (as an index of lipid peroxidation) after administration of bleomycin. The results of the present study also showed that administration of bleomycin induced a marked elevation in GSH-PX activity as well as GST activity.

It is documented that agents which cause lung damage through generating reactive oxygen species, stimulate the protective antioxidant cellular defense mechanism in order to detoxify the generated reactive radicals and lipid peroxides(Chandler et al.,1988 . Inghilleri et., 2006) .

The remarkable increase in the normal level of GSH-PX of the lung tissue after bleomycin treatment observed in the present study is in accordance with the finding of others who reported an elevation in the GSH-PX activity after bleomycin administration(Filderman et al., 1988, Arslan et al.,2002) .

Bleomycin toxicity was associated with increased GSH-shuttle enzymes (glutathione reductase and glutathione peroxidase) in response to oxidant stress induced by the drug treatment. On the other hand, it has been reported that GSH-PX activity was consistently reduced following treatment with bleomycin, this lowering of GSH-PX suggests an oxidative type of injury with bleomycin induced damage in lung tissue (Paranka and Dorr, 1994) . Furthermore, the decrease in GSH-PX has been reported to be potentially ascribable to inactivation by the increase in the reactive oxygen species or lipid peroxides when oxidative damage is extreme(Blum and Fridovich,1985; Hasegawa et al ., 1992;) . The discrepancy between the results of GSH-Px could be attributed to the difference in the schedule of treatment, severity of lung damage (injury vs fibrosis) and/or the time intervals adapted for the parameter measurement following bleomycin treatment. The observed increase of alkaline phosphatase activity in the lung tissue in the present study is similar to that reported by Narayanan , (2000) who reported a remarkable rise in the level of alkaline phosphatase in the

lungs injured by bleomycin .

In the present study, combined treatment of thymoquinone with bleomycin leads to a significant decrease in the bleomycin induced lung damage. The thymoquinone is a potent free radical scavenger, scavenging power being as effective as superoxide dismutase against superoxide (Nagi and Mansour ,2000). The free radical scavenging and anti-lipid peroxidation play part in the protective effect of thymoquinone against bleomycin induced lung toxicity. Our results are in agreement with recent studies that report the protective effect of thymoquinone against lung toxicity induced by other toxic agents like toluene (Kanter, 2011), while the recent data of our finding is the use of this protector with BL lung damage.

Also our results are consistent with alleviation of lung toxicity by other antioxidants such as caffeic acid phenethyl ester (Ozyurt et al., 2004) , N-acetylcysteine (Fu,et al ., 2010) alpha tocopherol (Deger et al., 2007) and Diallylsulfide (Kalayarasan et al., 2008).

It has been shown that THQ pretreatment protected other organs against oxidative damage induced by a variety of free radical generating agents, including carbon tetrachloride (Mansour et al ., 2001) and against chemical carcinogenesis (Nagi and Almakki , 2009).

The role of nitric oxide in bleomycin induced lung toxicity has been considered (Inghilleri et al.,2006). Nitric oxide has been reported to be involved in diverse physiological and pathophysiological processes including host immune defense, vasoregulation and the pathogenesis of diabetes (Corbett et al., 1992 and Nathan, 1992). Nitric oxide synthase (NOS), an enzyme that is involved in the synthesis of NO has been shown to be activated in the inflammatory lesion (Anderson et al., 1995 and Genovese et al., 2005). There are at least three types of NOS, the constitutive cNOS, the endothelial eNOS and the inducible iNOS (Nathan & Xie ,1994). Genovese et al., 2005, clearly demonstrate that iNOS plays an important role in the lung injury induced by bleomycin. The absence or inhibition of iNOS in mice (animals with genetic or pharmacological inhibition of iNOS) significantly prevent lung inflammation induced by bleomycin administration (Genovese et al., 2005)

In the present study, combined treatment aminoguanidine with bleomycin leads to significant decrease BL-lung damage. Aminoguanidine, an inhibitor of iNOS (Misko et al., 1993), was demonstrated to decrease the severity of the

pathophysiological changes attributed to excess NO production in diabetes (Li et al., 2011). Also, aminoguanidine had a protective role against gentamicin-induced acute renal failure (Polat et al., 2006). Our results are in agreement with other studies that report the protective effect of aminoguanidine against lung toxicity induced by bleomycin or other different lung injury models (Erglu et al., 2008 and Guo et al., 2009).

In conclusion, this study demonstrated that both thymoquinone and aminoguanidine significantly attenuate the development of lung toxicity. The results suggest that thymoquinone suppress iNOS expression due to its antioxidant and/or nuclear factor-Kappa B inhibitory property; however the effect of aminoguanidine was predominantly mediated by inhibition of iNOS activity, there by reducing peroxynitrite formation. We propose that the development of a more specific and potent inhibitors of iNOS might be beneficial in the prevention and treatment of lung toxicity.

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