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A Possible Modulatory Role of Nitric Oxide in Paraquat-induced Lung Injury in Mice

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Abstract: The present study was undertaken to evaluate whether stimulation or inhibition of Nitric Oxide (NO) synthesis could affect lung toxicity induced by acute administration of paraquat (PQ) in mice. L-arginine (L-arg.), N^G-nitro-L-arginine methyl ester (L-NAME) and aminoguanidine (AG) were employed as NO precursor, competitive and specific NO synthesis inhibitors, respectively. PQ was administered intraperitoneally to Swiss albino mice at a single dose of 50 mg kg⁻¹. L-arg. (700 mg kg⁻¹ day⁻¹), L-NAME (150 mg kg⁻¹ day⁻¹) or AG (100 mg kg⁻¹ day⁻¹) was given in drinking water of mice for 5 days before and one day after PQ administration. Appropriate controls were performed. PQ administration resulted in a pronounced elevation in lipid peroxides (157%) as well as decreased activity of alkaline phosphatase [ALP] (48%) and non-protein thiols (40%) in lung tissue compared to control non-treated mice as evidences of lung injury. Serum level of NO end products, nitrate and nitrite significantly elevated due to PQ administration (150%) as compared to control level. In mice given combined treatment of L-arg. and PQ, a remarkable rise in the serum level of nitrate and nitrite (140%) compared to the PQ group was observed. In addition, L-arg. ameliorated the increased level of lipid peroxides and non-protein thiols depletion as well as the decreased activity of ALP caused by PQ respectively. On the other hand, L-NAME and AG potentiated the deleterious effects of PQ on serum NO, lung lipid peroxides content, non-protein thiols content and alkaline phosphatase activity. In conclusion, PQ-induced lung injury in mice is alleviated by L-arg. but exacerbated by L-NAME and AG supplementation. This could point out to a possible protective role of NO in PQ lung toxicity.

Key words: Paraquat, L-arginine, L-NAME, aminoguanidine, nitric oxide, lung-toxicity

INTRODUCTION

Paraquat is a herbicide that highly toxic to all animals and humans^[1]. It is an oxidative stress inducing agent which generates free radicals, thereby elevating intracellular level of superoxide anion (O₂⁻). Paraquat accepts an electron by reductases and form paraquat radicals. These radicals transfer the extra electron to molecular oxygen forming superoxide anion radicals and regenerating the parent compound that is ready to gain a new electron^[2,3]. Superoxide radical changes to hydrogen peroxide by superoxide dismutase enzyme, in turn changes into hydroxyl radicals or detoxified by catalase or glutathione peroxidase in the presence of glutathione (GSH) to form water and oxidized glutathione (GSSG)^[3].

Nitric Oxide (NO) is a small membrane-permeable gas that is recently recognized as a physiological transmitter of diverse and vital functions. It synthesized from the amino acid L-arginine by an enzyme called the NO synthase (NOS). Two types of NOS have been

identified^[4]. One of them, the constitutive NOS (cNOS) is expressed under normal conditions and the NO released by this enzyme acts as transduction mechanism underlying several physiological responses. The other type of NOS, referred to as the inducible NOS (iNOS) is induced by certain cytokines such as lipopolysaccharide (LPS) and tumor necrosis factor- α ^[5].

It has been reported that NO can rapidly combine with superoxide to form peroxynitrite which results in nitric oxide scavenging. Peroxynitrite is a potent and versatile oxidant that can attack a wide range of biological targets. Superoxide dismutase by controlling superoxide levels, influence the reaction pathways open to nitric oxide^[6]. Meanwhile, NO has been implicated in PQ-induced lung injury^[7]. How and under what conditions NO can modulate tissue injury remains open question.

The present study was conducted to investigate the influence of oral supplementation with L-arg., the NO precursor, N^G-nitro-L-arginine methyl ester (L-NAME), a competitive NO synthase inhibitor or AG, a specific

inhibitor of the inducible NOS enzyme on lung injury induced by PQ administration.

MATERIALS AND METHODS

Chemicals: Paraquat (Methyl viologen), L-arginine, aminoguanidine hydrochloride, N-(1-naphthyl) ethylenediamine dihydrochloride (NEDD), sulfanilamide (SULF), vanadium trichloride, sodium nitrate and absolute methanol were all purchased from Sigma-Aldrich (St. Louis, MO, USA) and were used without further purification. N^o-nitro-L-arginine methyl ester (L-NAME) was provided by ICN Biomedicals Inc., Aurora, Ohio, USA. Thiobarbituric acid (TBA) was a product of Fluka (Buchs, Switzerland). Vanadium trichloride was stored in the dark under vacuum. All other chemicals were of the highest analytical grade and obtained from commercial suppliers. PQ was dissolved in normal saline just before use and was injected to mice in a volume not exceeding 0.2 mL L-arg., L-NAME and AG were dissolved in drinking water.

Animals: Male Swiss albino mice weighing 22-25 g were used in all experiments. They were obtained from the Experimental Animal Care Center, King Saud University, Riyadh, Saudi Arabia. They were housed under conventional laboratory conditions in a room temperature maintained at 25±1°C and a relative humidity range of 40 to 75% with a regular 12 h light:12 h dark cycle. Mice were fed a standard animal pellet diet and allowed free access to water unless otherwise indicated. Experiments were approved by a local ethical committee of College of Pharmacy, King Saud University, Saudi Arabia.

Experimental protocol: In this experiment, mice were randomly allocated into 8 groups, each consisting of eight animals. The first group, the control group (C), received saline. The 2nd group, L-arg. group, received L-arg. 2.8 g L⁻¹ in drinking water which is equivalent to 700 mg kg⁻¹ day⁻¹ p.o. for 5 days^[8,9]. A 3rd group, L-NAME group, received L-NAME 600 mg L⁻¹ in drinking water which is equivalent to 150 mg kg⁻¹ day⁻¹ p.o. for 5 days^[8,9]. The 4th group, AG group, was given AG 400 mg L⁻¹ in drinking water which is equivalent to 100 mg kg⁻¹ day⁻¹ p.o. for 5 days^[10]. The 5th group, PQ group, was treated with a single dose of PQ (50 mg kg⁻¹ i.p.)^[11]. The 6th, 7th, 8th groups were given L-arg. (700 mg kg⁻¹ day⁻¹ p.o.), L-NAME (150 mg kg⁻¹ day⁻¹ p.o.), AG (100 mg kg⁻¹ day⁻¹ p.o.) respectively for 5 days in drinking water and one day after a single dose

of PQ (50 mg kg⁻¹ i.p.). The doses were calculated according to the average daily water intake of mice.

Biochemical assessment of lung-toxicity: Before sacrificing the animals, blood samples were collected from the orbital venous plexus, under light ether anesthesia, into non-heparinized capillary tubes. Serum was separated by centrifugation for 5 min at 1000 g and stored at -20°C until analysis. Animals were sacrificed by cervical dislocation and the lungs were quickly isolated, washed with saline, blotted dry on a filter paper and weighed, then lung/body weight ratio was determined. Homogenization was carried out in ice-cold KCl (1.15%, pH 7.4) to yield a 10% (w/v) tissue homogenates using Glas-Col homogenizer (USA) then the following biochemical parameters were assessed.

Lipid peroxides (LP) level in lung homogenate was determined as thiobarbituric acid-reactive substances spectrophotometrically, the absorbance was measured at 532 nm by the method of Ohkawa^[12] and the concentrations were expressed as nanomole malondialdehyde (MDA) per gram tissue (nmol MDA g⁻¹ lung tissue).

Lung homogenate contents of acid soluble thiols mainly reduced glutathione were measured according to the method of Ellman^[13], the absorbance of the product was measured colourimetrically at 412 nm. Homogenates were precipitated with 5% perchloric acid and after centrifugation; supernatants were used for the estimation of GSH level. The concentration of GSH was expressed as μmol g⁻¹ lung tissue.

The activity of ALP enzyme was determined colourimetrically in lung homogenates at 510 nm^[14]. The results were expressed as μmol min⁻¹100 g⁻¹ lung tissue.

The serum level of total nitrate/nitrite (μmol mL⁻¹) was determined by the acidic Griess reaction after reduction of nitrate to nitrite by vanadium trichloride. The Griess reaction relies on a simple colorimetric reaction between nitrite, sulfonamide and N-(1-naphthyl) ethylenediamine to produce a pink azo-product with maximum absorbance at 543 nm. Prior to the Griess reaction all nitrate was converted to nitrite using vanadium trichloride as described^[15]. Serum proteins were precipitated by absolute methanol and after centrifugation, supernatants were used for estimation of nitrate/nitrite level.

Statistical analysis: Data were expressed as means±SEM. Statistical comparison between different groups were done using one-way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparisons test. Significance was accepted at p<0.05.

RESULTS

General observation: Administration of a single dose of PQ (50 mg kg⁻¹ i.p.) did not significantly alter body or lung weight of the treated mice. The ratio of lung weight/body weight also did not alter after treatment. Similarly, L-arg., L-NAME and AG or combined PQ and L-arg., L-NAME and AG treatment did not alter the body weight, lung weight or the ratio of lung weight/body weight.

Effect of L-arg., L-NAME and AG on PQ-induced changes in ALP activity in lung tissues: After PQ injection, ALP activity in lung homogenates was significantly decreased (48%) as compared with the control group reaching 52.3±1.4 μmol min⁻¹ 100 g⁻¹ lung tissue while control value was 109±9.8 μmol min⁻¹ 100 g⁻¹ lung tissue. Pretreatment with L-arg. (700 mg kg⁻¹ day⁻¹ p.o.) for five days before PQ injection prevented the significant reduction of ALP activity in lung homogenates while L-NAME (150 mg kg⁻¹ day⁻¹ p.o.) and AG (100 mg kg⁻¹ day⁻¹ p.o.) aggravated the PQ induced reduction in ALP activity. On the other hand, administration of L-arg., L-NAME and AG alone did not produce any significant change in the enzyme activity levels (Fig. 1).

Effect of L-arg., L-NAME and AG on PQ-induced changes in lipid peroxides and reduced glutathione content in lung tissues: Lipid peroxides in lung homogenates measured as malondialdehyde concentrations of mice treated with PQ significantly increased (157%) to 683±35.1 nmol g⁻¹ tissue as compared to control value of 435±11.3 nmol g⁻¹ tissue (Fig. 2). Pretreatment with L-arg. (700 mg kg⁻¹ day⁻¹ p.o.) for 5 days before PQ injection prevented the significant increase in lung malondialdehyde concentrations compared to PQ treated group while L-NAME (150 mg kg⁻¹ day⁻¹ p.o.) and AG (100 mg kg⁻¹ day⁻¹ p.o.) potentiated the effect of PQ on the MDA level. . On the other hand, PQ injection produced a significant reduction (40%) of reduced glutathione in lung homogenates (0.58± 0.01 μmol g⁻¹ tissue) as compared with the control value (1.46±0.08 μmol g⁻¹ tissue). Pretreatment with L-arg. (700 mg kg⁻¹ day⁻¹ p.o.) for 5 days before PQ injection prevented the significant depletion of lung GSH content compared to PQ treated mice while L-NAME (150 mg kg⁻¹ day⁻¹ p.o.) potentiated this depletion (Fig. 3).

Effect of PQ and/or L-arg., L-NAME and AG on serum nitric oxide [Nitrate + Nitrite] production: After a single dose of PQ injection (50 mg kg⁻¹ i.p.), serum nitrate + nitrite level was significantly increased (150%) as

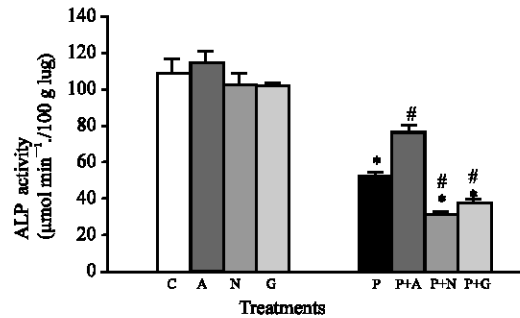


Fig. 1: Effect of L-arg. (700 mg kg⁻¹ day⁻¹ p.o.), L-NAME (150 mg kg⁻¹ day⁻¹ p.o.) and AG (100 mg kg⁻¹ day⁻¹ p.o.) on lung toxicity induced by PQ as measured by changes in ALP activity in lung tissue homogenates. L-arg., L-NAME and AG were administered orally for 5 days. On day 5, lung injury was induced in animals by a single i.p. injection of PQ (50 mg kg⁻¹ i.p.). One day thereafter, mice were sacrificed by cervical dislocation and lungs were rapidly excised and homogenized in chilled 1.15% KCl (pH 7.4) to yield 10% tissue homogenates. Data are expressed as means±SEM for all groups; n= 8. *, Significantly different from control group, #, Significantly different from PQ group (p<0.05). P indicates paraquat; ALP indicates alkaline phosphatase, A indicates L-arg., N indicates L-NAME and G indicates aminoguanidine

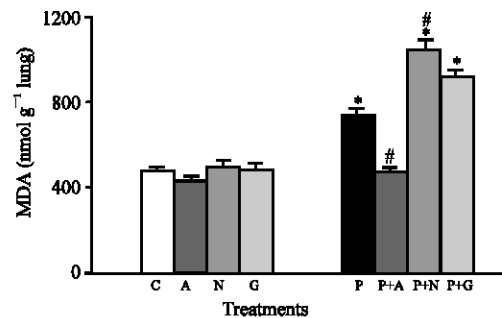


Fig. 2: Effect of L-arg. (700 mg kg⁻¹ day⁻¹ p.o.), L-NAME (150 mg kg⁻¹ day⁻¹ p.o.) and AG (100 mg kg⁻¹ day⁻¹ p.o.) on PQ induced enhancement of lung tissue MDA. *, Significantly different from control group, #, Significantly different from PQ group (p<0.05). P indicates paraquat; MDA indicates malondialdehyde, A indicates L-arginine, N indicates L-NAME and G indicates aminoguanidine

compared to the control group reaching 48.9±2.8 μmol mL⁻¹ while the control value was 32.6±1.6 μmol mL⁻¹

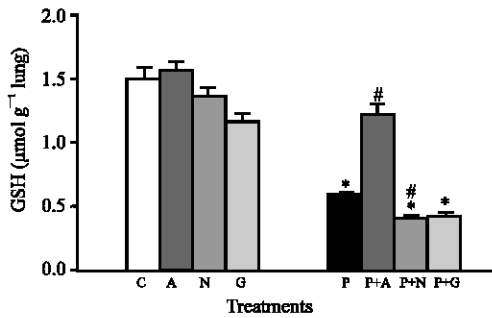


Fig. 3: Effect of L-arg. (700 mg kg⁻¹ day⁻¹ p.o.), L-NAME (150 mg kg⁻¹ day⁻¹ p.o.) and AG (100 mg kg⁻¹ day⁻¹ p.o.) on PQ-induced reduction of lung tissue GSH. *, Significantly different from control group. #, Significantly different from PQ group (p<0.05). P indicates paraquat; GSH indicates reduced glutathione, A indicates L-arginine, N indicates L-NAME and G indicates aminoguanidine

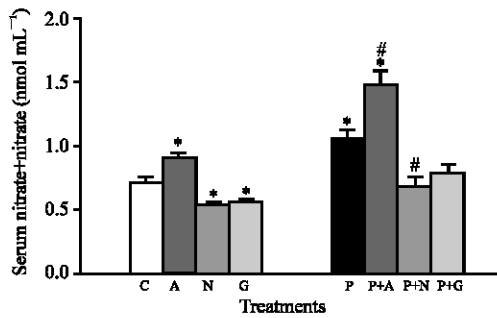


Fig. 4: Effect of L-arg. (700 mg kg⁻¹ day⁻¹ p.o.), L-NAME (150 mg kg⁻¹ day⁻¹ p.o.) and AG (100 mg kg⁻¹ day⁻¹ p.o.) on PQ-induced changes in serum nitrate + nitrite levels (nmol mL⁻¹). *, Significantly different from control group. #, Significantly different from PQ group. P indicates PQ, A indicates L-arg., N indicates L-NAME and G indicates AG

Pretreatment with L-arg. (700 mg kg⁻¹ day⁻¹ p.o.) for 5 days before PQ injection significantly increased the serum nitrate + nitrite level than that of PQ group (68±6.5 µmol mL⁻¹), on the other hand, L-NAME (150 mg kg⁻¹ day⁻¹ p.o.) significantly reduced serum nitrate + nitrite level nearly to the control value 32.7±1.6 µmol mL⁻¹.

DISCUSSION

Paraquat is a well known pneumotoxicant that exerts its toxic effect by elevating intracellular levels of

superoxide radical^[16]. The role of superoxide radical in paraquat-mediated cytotoxicity has been suggested from studies where overexpression of superoxide dismutase (SOD) or treatment with SOD mimetics is protective against PQ toxicity^[17]. In addition, nitric oxide has been implicated in PQ-induced lung injury^[7]. The mechanism by which nitric oxide modulates the PQ-induced lung injury still obscure. However, it thought that superoxide radical produced by PQ may react rapidly with NO to form the strong oxidant peroxynitrite^[18].

In the present study, intraperitoneal administration of PQ (50 mg kg⁻¹) significantly reduced the ALP activity in lung tissue homogenate to about 48% of the control value. This sharp drop in ALP activity may indicate lung damage as the decrease in ALP activity has been reported as a sensitive marker of the toxicity of the alveolar epithelium^[19]. This is in full agreement with previous investigations^[19-21]. Moreover, PQ caused depletion of non protein-thiols in lung tissue amounted to 40% of GSH of the control group. Meanwhile it produced a marked elevation of lipid peroxides measured as MDA lung homogenate to 157% of the control value. Excessive production of lipid peroxides is considered to be an indirect, *in vivo* feasible index for the contribution of free radical generation and in turn, to oxidative stress in the pathogenesis of several lung injuries, particularly those caused by exposure to exogenous oxidants^[22,23]. Previous reports documented that the increase in lung lipid peroxides is manifested in the oxidative damage provoked by paraquat exposure^[19-21,24,25].

In the present study, L-arg. (nitric oxide precursor), L-NAME (a competitive nitric oxide synthase inhibitor) and AG (specific inhibitor for iNOS) have been utilized as helpful tools to test whether stimulation or inhibition of endogenous nitric oxide synthesis, respectively, could affect lung toxicity induced by PQ. Results showed that L-arg., L-NAME and AG given orally to normal mice lead to significant alterations in the serum level of NO, determined collectively as nitrate + nitrite where L-arg. significantly increased serum nitrate + nitrite while on the other hand, both L-NAME and AG significantly decreased it as compared to the control group. It is obvious from the present study that L-arg. afforded an ameliorating influence on PQ lung toxicity, while L-NAME and AG (on the other hand) showed an opposite picture.

Present results showed that NO has a protective potential against PQ lung toxicity, since the co-treatment with L-arg. before and after PQ injection ameliorated the reduced activity of ALP and GSH depletion resulted from PQ injection alone, as well as it reduced the elevated lipid peroxides measured as MDA in lung tissue. This might explain the beneficial effect of NO inhalation in case of

PQ-increased lung toxicity^[26]. NO has paradoxical effects in oxidative stress models, where it can either be protective or injurious. Gutierrez and *et al.*^[27] reported that NO is protective to both pulmonary cells and animals in various models of oxidative stress. Present results suggest that PQ may use NOS as an electron source to generate superoxide radicals and in this process, decreasing the generation of NO.

It has been found that L-NAME (competitive NOS inhibitor) treatment significantly decreased NO production as compared to PQ injection alone and it aggravated the effect of PQ on GSH depletion as well as MDA elevation in lung tissue. Our results regarding L-arg. and L-NAME are in harmony with the findings of Gutierrez *et al.*^[27]. A similar pattern of modulation by L-arg.-NO pathway has been shown in various models of toxicity implicating free radicals attacks. L-arg. has been shown to attenuate bleomycin-induced lung toxicity^[9], carbon tetrachloride induced- chronic hepatotoxicity^[28] and cyclosporine induced-nephrotoxicity^[29]. L-NAME on the other hand was found to aggravate bleomycin-induced lung toxicity^[9], carbon tetrachloride induced-hepatotoxicity^[28], nephrotoxicity provoked by gentamycin^[30] and cyclosporine^[31]. Similarly, the combined treatment of aminoguanidine and paraquat produced an identical pattern to L-NAME. In conclusion, the data reported herein reveal that lung toxicity elicited by acute PQ administration is alleviated by L-arg. but exacerbated by L-NAME or AG supplementation. This could indicate a possible protective role of L-arg. and thus of NO against PQ-induced lung toxicity.

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