

Relationship between Pulmonary Surfactant Protein and Lipid Peroxidation in Lung Injury due to Paraquat Intoxication in Rats

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Background : Pulmonary damage resulting from lipid peroxidation is a principal effect of paraquat intoxication. The host-defense functions of surfactant are known to be mediated by the surfactant proteins A and D (SP-A and SP-D, respectively). The primary objective of this study was to evaluate the variations over time in levels of surfactant protein and lipid peroxidation (LPO) in lung tissue following free-radical-induced injury.

Methods : 42 adult, male, Sprague-Dawley rats were administered intraperitoneal injections of paraquat (35 mg/kg body weight). SP-A and SP-D levels were determined via Western blot. LPO in the left lung homogenate was measured via analyses of the levels of thiobarbituric acid-reactive substances.

Results : LPO levels peaked at 6 hours, with no associated histological changes. SP-D levels increased until hour 12 and declined until hour 48; SP-D levels subsequently began to increase again, peaking at hour 72. SP-A levels peaked at hour 6, declining thereafter.

Conclusions : We suggest that in the early phase of paraquat injury, SP-D levels reflect alveolar damage and that de novo synthesis of SP-D takes 72 hours. Levels of SP-A, on the other hand, reflect abnormalities in the surfactant system in the late stage of paraquat intoxication. Surfactant proteins may play a role in protecting the lungs from reactive oxygen injury. A time-dependent variation has been observed in the levels of surfactant proteins A and D following paraquat injury, and it has been suggested that these proteins play a role in the protection of lung tissue against ROS-induced injuries.

Key Words : Paraquat, Lipid peroxidation, Surfactant protein A, Surfactant protein D

INTRODUCTION

Paraquat dichloride (1,1'-dimethyl-4,4'-bipyridilium dichloride; methylviologen) is an effective and widely-employed herbicide. Paraquat is toxic in humans, with the lungs being the principal target organ, probably as the consequence of the existence of an active uptake system^{1,2)}. The toxic effects of paraquat on the lungs result in pulmonary edema, hypoxia, respiratory failure, and

pulmonary fibrosis. Mortalities as the result of paraquat poisoning are generally attributed to extensive pulmonary injury³⁾. Respiratory distress occurring during the early stages of paraquat poisoning may be attributable to impairments of the surfactant system, probably resulting not only from the paraquat itself, but also from the effects of oxygen free radicals. Pulmonary fibrosis in the late stage of paraquat poisoning is generally observed 531 days after poisoning. Manktelow demonstrated that paraquat induces

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changes in the lung that are comparable to the lesions observed in cases of respiratory distress syndrome⁴⁾. Pulmonary surfactant is composed of 90% lipid and 10% protein. Four surfactant-associated proteins exist: surfactant protein A (SP-A), surfactant protein B (SP-B), surfactant protein C (SP-C), and surfactant protein D (SP-D). Evidence has accumulated over the past 1015 years that SP-A and SP-D function as part of the first line of immune defense in the lung, by binding to pathogens, thereby promoting their phagocytosis and killing by phagocytes⁵⁾. SP-A-knockout mice evidence normal lung histology, but lack SP-A, as has been predicted, exhibiting an increased susceptibility to pulmonary infection by bacteria and viruses⁶⁾. By way of contrast, murine SP-D deficiency unexpectedly induces spontaneous emphysematous change and the development of pulmonary fibrosis, thereby revealing the crucial role for SP-D in the control of lung inflammation^{7, 8)}. The pathology and biochemical characterization of paraquat-induced lung-injury models have been previously reported^{9, 10)}. The present study was undertaken in order to characterize the variations over time in surfactant protein levels, and to determine the levels of lipid peroxidation in lung tissue following free-radical-induced injury.

MATERIALS AND METHODS

Animal preparation

Adult male Sprague-Dawley rats (approximate body weight 200–220 g), which were maintained on a standard laboratory diet and water, were employed in this study. The rats received intraperitoneal (ip) injections with paraquat (35 mg/kg body weight, Sigma Chemical, St. Louis, MO, USA) in 1 ml saline. The animals were anesthetized via ip injections of phenobarbital (50 mg/kg body weight) at time 0 (control, immediately after paraquat injection), 6 hours, 12 hours, 24 hours, 2 days, 3 days, or 5 days after the paraquat injection. They were then exsanguinated via the abdominal aorta, and the chest wall was opened.

Alveolar lavage

Three aliquots of 7.5 mL saline at 4°C were used to fill the lungs. Each aliquot was flushed into and out of the airways three times. The recovered volumes (about 85% of the injected volume) from all animals from the same experimental group (control, and 6 hours, 12 hours, 24 hours, 2 days, 3 days, or 5 days postinjection) were pooled.

Total protein assay

Protein concentrations in the lavage fluid were measured via the Bradford method¹¹⁾ using bovine serum albumin as a standard.

Preparation of rat lung bronchoalveolar lavage (BAL)

BAL fluid was collected from paraquat-treated rats and diluted to 15 mL in BNC buffer (10 mM sodium borate, pH 7.4, 150 mM NaCl, 3 mM CaCl₂). The sample was centrifuged for 10 minutes at 250 *g* at 4°C, followed by 2 hours of centrifugation at 27,000 *g*, and the resultant pellet was resuspended in 100 l of BNC buffer and stored at 20°C. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western blot analysis were then conducted. Samples containing 1 g of BAL were boiled for 4 minutes and applied to 12% NuPAGE Novex Bis-4,7-diphenyl-1,10-phenanthroline (Tris) gels using the XCell SureLock Mini-Cell system (Invitrogen Life Technologies), in accordance with the manufacturer's instructions. After electrophoresis, gels were transferred onto polyvinylidene difluoride membranes at 35 V for 2 hours, followed by 1 hour of blocking in phosphate-buffered saline (PBS) with 0.5% bovine serum albumin and 0.02% Tween 20. The membranes were then incubated overnight at 4°C in a primary antibody against either SP-A or SP-D (Santa Cruz) diluted to 1:500 with PBS. The membranes were then incubated for 1 hour in peroxidase-conjugated secondary antibody diluted to 1:1,000 with PBS at room temperature. The blots were developed with an enhanced chemiluminescence detection system (ECL Plus from Amersham Bioscience)¹⁰⁾.

Left lung homogenate and determination of tissue thiobarbituric acid-reactive substances (TBA-R S) contents

After the lungs were perfused free of blood and harvested for lavage fluid, the left lung was placed in a tube and rapidly frozen in enzyme-linked immunosorbent assay buffer and then stored at 70°C. For analysis, the tissue was placed in homogenizing buffer (50 Mm Tris HCl, pH 7.5, containing 1 Methylene diaminetetraacetic acid, 2 mM phenylmethylsulfonyl fluoride, and 2.5 mM N-ethylmaleimide) at a defined ratio of 1 g of lung tissue to 9 mL of homogenizing buffer. The lung tissue was then homogenized on ice with a Polytron (Brinkman Instruments, Westbury, NY, USA). The lung homogenates were spun for 5 minutes at 300*g* to sediment the tissue debris. The fluorometric method of Okhawa, (excitation at 532 nm; emission at 551 nm) was utilized in order to determine the tissue TBA-RS contents¹¹⁾. A standard curve was prepared with the aid of tetramethoxypropane (Sigma), which under the assay conditions is hydrolyzed to malondialdehyde (MDA). The detection limit was 0.8 mmol MDA-thiobarbituric acid/mL. TBA-RS levels are expressed as nmol/g of tissue. A light microscopy procedure was then performed. The rats were anesthetized with sodium phenobarbital, after which a thoracostomy and BAL were conducted, and the right lung was sectioned. The right lung was fixed with an intratracheal instillation of 4% formaldehyde and 1% glutaraldehyde in 0.1 M phosphate buffer at a pH of 7.2 at room temperature. For light microscopy, the pulmonary tissue blocks were embedded in paraffin and sectioned at 4 μ m. The sections were mounted on glass slides and

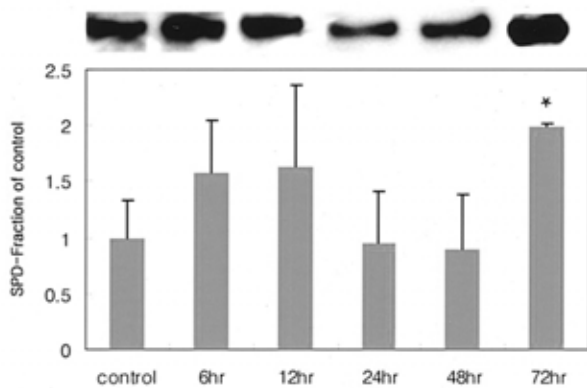


Figure 1. Variations in the levels of surfactant protein D (SP-D) over time in rats treated with paraquat. SP-D was quantified via Western blot analysis and data were expressed as a fraction of that measured in the control group. Data are expressed as the meanSD values. No significant differences were detected in the initial SP-D levels among the groups (as assessed by ANOVA). The SP-D level in the 72-hour postinjection group was significantly higher than those of the control, and the 24-hour and 48-hour postinjection groups (* ; $p < 0.05$) (Student's *t*-test).

stained with hematoxylin and eosin.

Statistical analysis

Data are expressed as meansSD. A probability value of $p < 0.05$ was considered to be indicative of statistical significance, with all statistical analyses conducted using SPSS for Windows (version 12.0, Chicago, Illinois, USA). In cases in which an *F* test indicated homogenous variances, the Student's *t*-test was applied; if not, a modified Student's *t*-test was utilized. Differences between groups were tested via ANOVA. For multiple comparisons, Tukey's test was applied after ANOVA.

RESULTS

Mortality and clinical observations

Eight of the 42 paraquat-treated rats died before sacrifice. The timing and number of deaths were as follows: <24 hours, 0; 24-48 hours, 2; 48-72 hours, 2; >72 hours, 4). Some of the paraquat-treated rats evidenced hypokinesia and anorexia.

SP-D levels

As is shown in Figure 1, SP-D levels in the lung lavage increased until hour 12 after the paraquat injection. At 24 hours, SP-D levels again declined to near control levels, but subsequently increased again, achieving peak levels at 72 hours. Differences between groups were not statistically significant ($p = 0.66$). SP-D levels were higher at 72 hours' posttreatment than

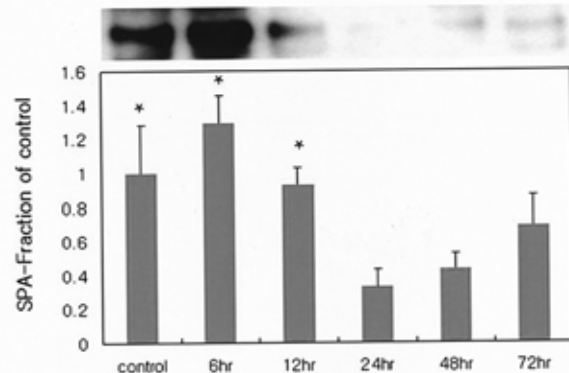


Figure 2. Variations in the levels of surfactant protein A (SP-A) over time in rats treated with paraquat. SP-A was quantified via Western blot analysis and data are expressed as a fraction of that measured in the control group. Data are presented as the meanSD values. Differences between groups were statistically significant (as assessed by ANOVA): SP-A levels in the control, and 6-hour and 12-hour postinjection groups were significantly different from those of the 24-hour, 48-hour, and 72-hour groups (* ; $p < 0.05$) (Tukey's test).

in the control animals, or at 24 hours and 48 hours posttreatment ($p < 0.05$).

SP-A levels

As is shown in Figure 2, SP-A levels in lung lavage fluid achieved peak levels at 6 hours after paraquat injection, after which they declined to levels below normal (control). Differences between groups were statistically significant ($p < 0.05$) as follows: control, 6-hour, and 12-hour posttreatment groups were significantly different from the 24-hour, 48-hour, and 72-hour posttreatment groups (Tukey's test).

Lipid peroxidation

Tissue TBA-RS contents differed significantly between the groups, achieving peak levels at 6 hours' postinjection. TBA-RS levels in the control group differed significantly from those of the 6-hour postinjection group ($p < 0.05$) (Figure 3).

Light microscopy

As is shown in Figure 4, the lung tissue in paraquat-treated rats evidenced inflammatory cell infiltration around the bronchioles at 12 hours' postinjection. Inflammatory cell infiltration and hemorrhaging were noted 72 hours after paraquat injury.

DISCUSSION

Paraquat is a widely used and effective herbicide, which

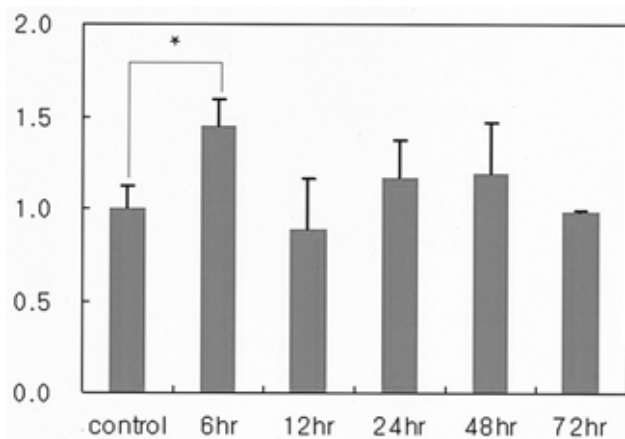


Figure 3. Variations in the levels of lipid peroxidation over time in rats treated with paraquat. Concentrations of thiobarbituric acid-reactive substances (TBA-RS) in left lung tissue, expressed as a fraction of that measured in the control group. Data are expressed as meanSD values. Differences between groups were statistically significant (as assessed by ANOVA): levels of lipid peroxidation in the control group differed significantly from those measured in the 6-hour postinjection group (* ; $p < 0.05$) (Tukey's test)

evidences a broad spectrum of activity. Experimental studies have indicated that it accumulates in the epithelial cells of the lung and kidney, eventually resulting in pulmonary fibrosis and acute renal failure. Lung damage after paraquat poisoning appears to be secondary to derangement of the pulmonary surfactant system^{4, 12-14}, probably resulting not only from selective paraquat-induced injury to type 1 and type 2 cells, but also from the increased permeability of alveolar capillaries after oxygen-radical-induced injury to endothelial cells¹⁵. The characteristic proliferative lesions associated with paraquat poisoning, namely pulmonary fibrosis, may not become evident until 1 week after injury, whereas damage resulting from free radicals commences immediately¹⁶. The destructive phase is characterized by the loss of type I and type II alveolar cells, loss of surfactant, infiltration by inflammatory cells, and hemorrhaging. The subsequent proliferative phase is characterized by a loss of alveolar space. The fibrosis, which is mediated in part by cytokines, is not specific to paraquat-induced injury, but rather is observed in response to the acute alveolitis induced by a variety of pulmonary toxins¹⁷.

The overwhelming of tissue defense mechanisms by excessive

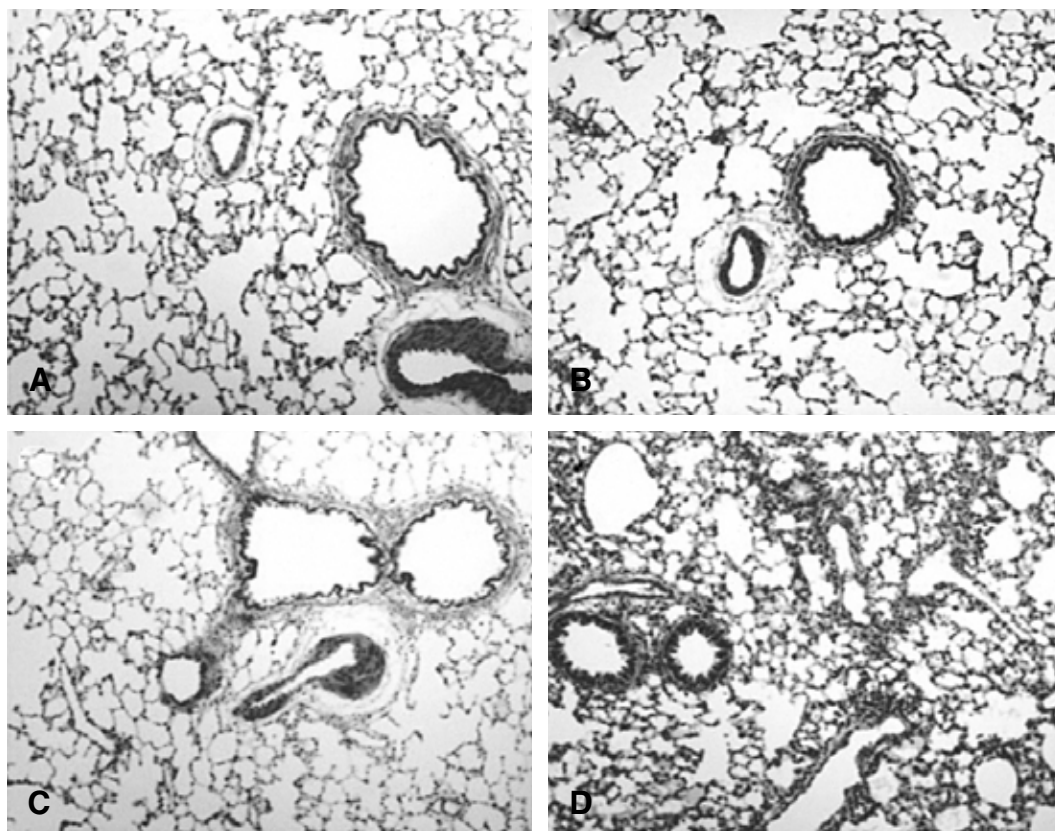


Figure 4. Light micrograph showing the inflammatory cell infiltration occurring after paraquat injection (hematoxylin and eosin, 100). The control (A) and 6-hour postinjection groups (B) evidence normal cytoarchitecture. The 12-hour postinjection group (C) exhibits inflammatory cell infiltration around the bronchiole. The 72-hour postinjection group (D) exhibits diffuse lung damage on the parenchyma and peribronchiole.

free-radical production may induce a lipid peroxidation process, thereby resulting in extensive membrane damage. Several markers have been proposed for the detection of lipid peroxidation, but measurements of TBA-RS are probably the most widely used technique. In the present study, measurements of tissue TBA-RS levels showed that oxidative injury began at an early stage, at which point it induced no histological changes. It has been established that the surfactant system is one of the targets of paraquat toxicity. In a variety of animal studies, inhalation of surfactant proved effective in preserving lung compliance after paraquat poisoning¹⁸. Surfactant is composed principally of phospholipids, which are essential for reducing surface tension at the air-liquid interface of the lung. Approximately 10% of surfactant is protein, and four surfactant proteins have been identified: SP-A, SP-B, SP-C, and SP-D. SP-B and SP-C are small and extremely hydrophobic. SP-B is crucial for the ability of surfactant to reduce surface tension¹⁹. The host-defense functions of surfactant are mediated principally by SP-A and SP-D, both of which are members of the collectin protein family²⁰.

In the study presented herein, the concentration of SP-D in the lung was elevated until 12 hours after the paraquat injection. After 1 day, the level of SP-D had declined to below control levels; they then began to increase again, achieving peak levels at 72 hours' postinjection. We suggest that in the early phase after paraquat injury, the level of SP-D is reflective of the alveolar damage induced by excessive free-radical production. During this phase, the SP-D levels measured may originate from endogenous stores; *de novo* synthesis of SP-D takes 72 hours. By way of contrast, SP-A levels peaked at 6 hours' postinjection, subsequently declining to below control levels. This indicates that SP-A, which is intimately associated with surfactant lipid membranes and aggregates²¹, performs a role different from that suggested by the lung model of paraquat injury. Experimental data imply that SP-A preserves the surface activity of surfactant, protects it against the effects of serum protein inhibitors²², contributes to the stability of surfactant aggregates including tubular myelin²³, and inhibits phospholipase A2 activity^{24, 25}. SP-D, however, appears to be a prerequisite for the maintenance of surfactant homeostasis and lung structure⁹. In the study presented herein, the effects of paraquat on SP-A levels began only after 6 hours' post-injection, thereby suggesting that this surfactant protein is affected in the late stage after paraquat intoxication.

Several factors may be responsible for the variable effects of paraquat intoxication on SP-A and SP-D over time. First, SP-A and SP-D function as endogenous antioxidants. Paraquat elicits the generation of reactive oxygen species (ROS), which destroy type II pneumocytes. The elevation of surfactant protein levels may protect the lung tissue against paraquat-induced ROS. Bridges previously demonstrated that pulmonary SP-A and SP-D are potent endogenous inhibitors of lipid peroxidation and

oxidative cellular injury²⁶. SPD attenuates alveolar macrophage apoptosis *in vivo*²⁷, evidences antioxidant properties, and is crucial to the modulation of apoptotic cell numbers in the lung, thereby suggesting a protective function for this surfactant protein in the prevention of abnormal alveolar remodeling after oxidative lung injury, and may also play a role in immune and lung developmental processes.

Second, in the early stages of paraquat intoxication, SP-A and SP-D levels reflect the extent of lung injury. Pan previously demonstrated that serum SP-D is a marker for lung injury in rat²⁸. In cases in which paraquat, bleomycin, HCl, or keratinocyte growth factor are instilled into the lungs, serum and pulmonary levels of SP-D are elevated in BAL fluid. SP-D levels may reflect the extent of lung injury in the early stages of paraquat poisoning. In the later stages, levels of SP-A may be the result of destructive lung injury and fibrosis. Exogenous surfactant therapy has proven effective in several animal models of acute lung injury^{29, 30}, and has also been evaluated in humans suffering from sepsis-induced acute respiratory distress syndrome (ARDS).

However, clinical responses to exogenous surfactant therapy have varied substantially. A large multicenter clinical trial using aerosolized artificial surfactant was not determined to benefit patients with sepsis-induced ARDS²⁹. The lack of response to artificial surfactant may have been resultant from the absence of surfactant apoprotein. In the paraquat-induced lung injury model, exogenous surfactant tended to increase the survival rate and reduce pulmonary surface wall tension in rats. In humans, a commercial surfactant (survanta) may prove inadequate to treat patients with paraquat intoxication, as it does not include SP-D.

Supplementation with surfactant proteins may therefore prove efficacious in patients in the early stages of paraquat intoxication.

Our results demonstrate that there is a time-dependent variation in the levels of surfactant proteins after paraquat injury, and suggest that these proteins perform a function in the protection of lung tissue from ROS-induced injury.

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