PULMONARY MORPHOMETRIC HISTOPATHOLOGY OF SALINE OR PARAQUAT-EXPOSED RATS TREATED WITH THE COLLAGEN INHIBITOR DEHYDROPROLINE

by

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DEDICATION

To my friend, Dima Hamarneh.

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I extend, finally, my appreciation to my mum, dad and brother, John for their moral support during the course of this study.

INTRODUCTION

Paraquat is a non-selective herbicide. The basic chemical nucleus of paraquat is a bipyridyl consisting of two pyridine rings joined together in such a way that their nitrogen atoms face diametrically away from each other (Fig 1). Hence, paraquat is a quaternary bipyridyl. Since each nitrogen atom bears a methyl group side chain in the para position (Fig 2), the chemical is therefore, a PARA-substituted QUATernary bipyridyl--hence the common designation PARAQUAT (1). Other closely related analogues of paraquat are diquat (1,1'-ethylene-2,2'-dipyridylium dibromide) (Fig 3) and morfamquat (1,1'-(3,5'-dimethylmorpholinocarbonyl)-4,4'-dipyridilium dichloride) (Fig 4).

In many countries where paraquat has been used as a herbicide, deaths of many people have been reported and associated with their exposure to paraquat. The most devastating effect of paraquat poisoning occurs in the lungs where it initially trigers exudation of edema fluid and hemorrhages into the lungs parenchyma (2). If the victim survives these initial effects, a fulminating fibrotic process develops (2) and death results from anoxia due to progressive pulmonary fibrosis. The toxic manifestations and processes appear to be similar in almost all animals including man. Extensive necrosis of the pulpulmonary alveolar epithelium with the attendant inflammatory changes has been known to be caused by paraquat (3), but the resulting interstitial fibrosis of the lungs has not been seen in rabbits and

guinea-pigs (4,5). This may suggest resistance of these experimental animals to experimentally-induced paraquat poisoning.

Paraquat has been shown to function as a redox agent and contributes to the formation of an oxygen free radical (6,7). Paraquat causes lipid peroxidation which damages cell structure and function by destroying the membranes of the mitochondria, microsomes and other subcellular organelles. Since it is generally accepted that the action of paraquat as a herbicide is most likely due to lipid peroxidation, many experiments have been carried out to determine the effectiveness of free radical scavengers and anti-inflammatory agents (2,8), superoxide dismutase (9,10) and vitamin E (6) as prophylactic or therapeutic agents in the treatment of paraquat intoxication. However, their use has not been clinically effective in spite of the fact that they seemed to have protective effects in experimental studies (2,6,11). There is no therapeutic agent presently known to have an antidotal effect in paraquat poisoning.

Paraquat-poisoned rats have increased levels of hydroxyproline, increased prolyl-hydroxylase and transglutaminase activity in the lungs (12), and an increased rate of collagen synthesis (10,13). These increases in enzyme activity and collagen synthesis correspond to the early histological appearance of cellular proliferation in the lung alveoli (14).

The proline analogue dehydroproline (DHP) inhibits the biosynthesis and secretion of collagen (15). Of the several proline analogues tested in vitro, DHP is the most potent inhibitor of

aminoacylation reactions, particularly the aminoacylation of prolyl-t-RNA (15). Dehydroproline is a competitive inhibitor which can serve as substrate in this aminoacylation reaction. The administration of DHP also inhibits prolyl-hydroxylase activity (16), and although the exact mechanism is not certain, DHP can form stable complexes with prolyl-hydroxylase in vitro (17). In addition to its effects on biosynthesis, DHP has been shown to inhibit the secretion of both tropoelastin and collagen (18). However, no studies have been found on the ability of DHP to inhibit the elevated enzyme activities which occur following acute lung injury.

This project investigates the effectiveness of L-3,4-dehydroproline (L-DHP) and DL-3,4-dehydroproline (DL-DHP) as therapeutic agents for treating lungs from paraquat-exposed rats by comparing the degree of fibrosis and consolidation of the lung parenchyma in rats that received saline/saline or paraquat/saline to those that received paraquat/L-DHP or paraquat/DL-DHP. The reaction of paraquat intoxication in man and domestic animals is similar to that seen in rats in which the oral LD_{50} to paraquat is 150 mg/kg weight.

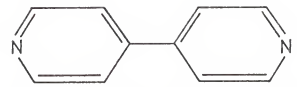

Fig. 1: Basic Chemical Nucleus in Paraquat.

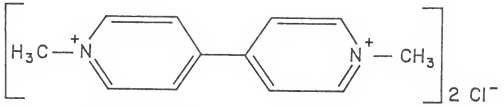

Fig. 2: Paraquat Dichloride.

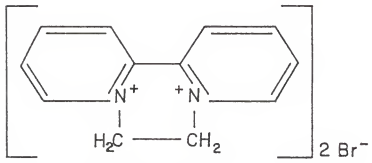

Fig. 3: Diquat Dibromide.

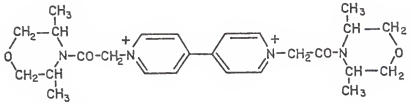

Fig. 4: Morfamquat.

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POLMONARY MORPHOMETRIC HISTOPATHOLOGY OF SALINE OR PARAQUAT-EXPOSED RATS TREATED WITH COLLAGEN INHIBITOR DEHYDROPROLINE

Key Words

Paraquat

Morphometric histopathology

Dehydroproline

Collagen inhibitor

Pulmonary interstitial fibrosis

Pulmonary consolidation

Proliferation

Type I and type II pneumocytes

Fibroblast

Macrophages

Footnotes

[a]Sigma Chemical Corporation, St. Louis, MO.

[b]Dr. Richard Salvador, Hoffman-La Roche, Nutley, New Jersey.

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Summary

Exposure to the herbicide paraquat has caused animal and human deaths due to pulmonary edema, hemorrhage, increased collagen synthesis and progressive pulmonary fibrosis. Because dehydroproline (DHP) inhibits the biosynthesis of collagen, its value as an antidote for paraquat poisoning was studied in rats.

In a previous study,[6] subcutaneous administration of 7 mg paraquat/kg body weight for 7-8 days caused early pulmonary fibrosis and a decrease in body weight 1-10 days after paraquat dosing was started. L-3,4-dehydroproline, DL-3,4-dehydroproline or saline was then administered to three randomized groups of the paraquat-dosed rats at 25 mg/kg body weight for 14 days (days 11-24). One group of rats that earlier recieved no paraquat, but only saline, was treated with saline as a negative control group. Representative number of rats for each group were sacrificed on days 10, 18, 25, 32, 39 and 100. Lung tissue from this experiment[6] was collected and fixed in formalin for morphometric histopathological measurements. The following parameters were quantitated by light microscopy: number of intra-alveolar macrophages and leucocytes/fibroblasts, total number of pulmonary cells, mean alveolar wall thickness, percent consolidation of lung parenchyma and the relative number of various alveolar cell types (types I and II pneumocytes). The results were compared between the various groups using analysis of variance.

Dehydroproline significantly reduced the number of pulmonary cells, especially the number of intra-alveolar macrophages. If stimulation of fibroblastic activity is through the medium of macrophage numbers, DHP decreased the lung response to paraquat exposure. However, the administered DHP did not significantly reduce the accompanying pulmonary fibrosis or consolidation.

Introduction

Paraquat dichloride (Fig 1) causes a fulminating fibrotic process in the lungs; death occurs in several days from anoxia due to the progressive pulmonary fibrosis.[1,2] Initially, exudation of edema fluid and hemorrhages occur in the lung parenchyma. Paraquat is a redox agent and contributes to the formation of oxygen free radicals.[3,4] These radicals cause lipid peroxidation which damages cell membranes and subcellular organelles.[3] Although many experiments have studied the effectiveness of free radical scavengers as prophylactic or therapeutic agents for paraquat intoxication, their clinical use has not been effective.[5]

Additional efforts have been made using biochemical and morphological approaches to evaluate the primary damage due to paraquat and devise an effective treatment.[6,7,8,9] Paraquat-poisoned rats have increased lung levels of hydroxyproline and prolyl-hydroxylase and an increased rate of collagen synthesis, leading to pulmonary fibrosis.[10] The proline analogue, dehydroproline (DHP), inhibits the biosynthesis and secretion of collagen by competitively blocking the aminoacylation of t-RNA by proline; DHP also inhibits prolyl-hydroxylase activity.[1,11] Because of these inhibitory effects on lung collagen biosynthesis, DHP was studied as a possible therapeutic agent for experimental paraquat poisoning in rats. Specifically, the effectiveness of the proline analogues L-3,4-dehydroproline (L-DHP) and DL-3,4-dehydroproline (DL-DHP) was examined.

In a preliminary study,[6] it was determined that the subcutaneous administration of 7 mg paraquat dichloride/kg body weight for 7-8 days initiated lung damage appropriate for the study of biochemical lung changes. Paraquat-exposed rats were then treated with either saline or 25 mg DHP/kg body weight for 14 days. Representative numbers of rats from each group were then sacrificed periodically for up to 100 days, and the protein and collagen content of the lungs were determined. No significant difference in collagen deposition was found in the lungs of rats treated with saline versus those treated with DHP.

To further study the effect of DHP on the lung reactions in paraquat-exposed rats, morphometric histopathological measurements of formalinized lung tissues from this experiment[6] were performed under light microscopy. This report presents the result of those measurements.

Materials and Methods

Animals and drug administration--The one hundred and thirty four, six-week-old male Sprague-Dawley rats in this experiment weighed between 135-218 g, and were obtained from the Animal Resources Facility of Kansas State University's College of Veterminary Medicine. After 14 days of observation for acclimation, the rats were matched by weight and allocated randomly into saline-dosed (n=31) or paraquat-dosed (n=103) groups. The 31 control (saline-dosed) rats received 1.4 ml/kg body weight of sterile saline solution and the other 103 rats received 7 mg paraquat dichloride[a]/kg body weight for 7 or 8 days (Fig 2). All treatments were given subcutaneously. Ten days after the start of paraquat dosing (day 10), the paraquat-dosed group of rats were divided

randomly into three groups to receive L-DHP[b] (paraquat/L-DHP) or DL-DHP (paraquat/DL-DHP) or saline (paraquat/saline); 34 rats received 25 mg L-DHP, 34 rats received 25 mg DL-DHP/kg/day, and 35 rats received 1.4 ml sterile saline/kg/day, each for 14 consecutive days (days 11-24) (Fig 2). The saline-dosed group of rats also received saline injections for days 11-24 (saline/saline).

Collection and preparation of lung tissue--Rats from the four groups (saline/saline, paraquat/L-DHP, paraquat/DL-DHP, paraquat/saline) were sacrificed on days 10, 18, 25, 32, 39 and 100 after the paraquat dosing was started (Fig 2) to collect lung tissues for morphometric histopathology. Sacrifice was induced using methoxyflurane (2,2' Dichloro-1,1-difluroethyl methyl ether) and the rats were drained of blood via the abdominal arota. The lungs were removed by clasping the trachea and lifting the organ out. The lungs were then blotted lightly on a paper towel and the right posterior part of the lobe of the lung was fixed in 10% buffered neutral formalin (BNF). The lung tissues were sectioned to 6 um thickness and stained with hematoxylin and eosin (Harris method).

Morphometric measurements of the lung sections--Various morphometric parameters were measured using a Zeiss RA research microscope with eyepiece micrometer calibration. One division of the eyepiece micrometer calibration equaled 0.98 um with 100x objective and 10.0x ocular (oil-immersion lens). The area of the field of view of the oil-immersion lens was calculated using the formula of the surface area of a circle, $4\pi r^2$, or πd^2. Using the eyepiece micrometer, the

radius of the field of view was approximately 0.08 mm and the field of view area was calculated to be 0.08 mm^2. The values obtained for the various morphometric parameters using this technique were averages from 30 field measurements per parameter for every 0.08 mm^2 field of view.

The following parameters were measured:

Number of intra-alveolar macrophages. These macrophages were phagocytic cells found within the alveolar spaces and characterized by rounded appearances of the cells with irregular cytoplasmic membranes. The nuclei were small but rich in chromatin (heterochromatic), and their cytoplasm took on a foamy appearance. For the purpose of this investigation, epithelioid and giant cells were also considered as macrophages.

Number of intra-alveolar leucoctyes/fibroblasts. These included white blood cells involved in the inflammatory response and fibroblasts, since in paraquat poisoning the offending cells appeared to be predominantly fibroblasts infiltrating into the alveolar spaces. Inflammatory leucocytic cells were minimal in number.

Total number of pulmonary cells. This was the summation of the macrophages and leucocytes/fibroblasts within the alveolar spaces, but did not include the alveolar epithelial cells.

Mean alveolar wall thickness (in micrometer, μm). The alveolar wall comprised the sparse connective tissue between the basement membranes of adjacent alveoli.

Percentage consolidation of lung parenchyma. This constituted that part of the lung parenchyma which had become filled with inflammatory cells, exudates, necrotic epithelial cells and other debris, and was expressed as a percentage of the lung tissue measured. The percent consolidation was determined using a microprojector. In this technique the affixed specimen was projected on to a 100 cm^2 white area background and the percentage of the total lung sample that was consolidated determined (grid estimation[12]).

Relative number of cell types (types I and II pneumocytes) in percentages. The more numerous type I pneumocytes were squamous, and interspersed between these were the cuboidal type II pneumocytes. Both of these cell types sat on the alveolar basement membrane. In routine hematoxylin-eosin stained sections, the basement membrane showed a thick reticular layer.

The areas measured for all these parameters were randomly selected alveolar fields. Some of the areas were photographed using Kodak Panatomic X film.

Statistical analysis--The data were analyzed by analysis of variance (ANOVA) using a Statlab Harris 80 Computer with a two-way treatment structure (treatment and time) in a completely randomized design. To determine if there was any significant differences between the paraquat/DL-DHP and the paraquat/saline rats allowed to survive to day 100 (Fig 2), the group differences in these instances were compared to a "critical value" (i.e. the value the group differences must exceed to be of any statistical significance for each parameter under

consideration). In all analyses, significant differences were established at the $p<0.05$ level.

Results

Alveolar histopathological impressions--The lungs and alveolar structure from the rats in the saline/saline group remained normal throughout the study (Fig 3). The groups that were exposed to paraquat had typical paraquat alveolar reactions (Figs 4 and 5) immediately after the paraquat-exposure period (day 10). This reaction of cellular infiltration, alveolar wall thickening and lung consolidation increased in severity with time following the paraquat exposure despite DHP treatment (Fig 6). The rats that received paraquat and then saline as a positive control group had especially severely affected lungs that had extensively thickened alveolar walls and consolidation of the parenchyma, as well as marked macrophage, leucocyte and fibroblast infiltration. By day 39 after initiation of the paraquat exposure these microscopic pathological lesions were severe (Fig 7).

Number of intra-alveolar macrophages--The average number of intra-alveolar macrophages remained constant in the saline/saline negative control group and in the paraquat/DL-DHP group, while they decreased in number in the paraquat group treated with L-DHP and increased in number in the paraquat positive control group that received only saline during the treatment period (Table 1). This trend continued in the paraquat/DL-DHP and paraquat/saline groups into day 100.

Number of intra-alveolar leucocytes/fibroblasts--The paraquat/L-DHP group lung had a significant ($p<.0001$) decline in the number of leucocytes and fibroblasts after day 10 when the treatment was given (Table 1). For instance, there was a decrease from 6 to 3 leucocytes/0.08 mm^2 field of view from day 10 to day 18. The other groups had no significant changes.

Total number of pulmonary cells--The rats that received paraquat and then L-DHP had a significant decrease in the total number of pulmonary cells e.g., a decline from 29.6 to 18 cells/0.08 mm^2 from day 10 to 18 ($p<.05$) compared to the paraquat/saline group, which had an increase in the number of pulmonary cells from 13.3 to 23 cells/0.08 mm^2 on these days.

Alveolar wall thickness--No statistically significant difference existed between the various treatment groups of the paraquat-exposed animals; all had marked increases in the mean alveolar wall thickness, when compared to the saline/saline control group ($p<.05$), that was not affected by the treatments given.

Percent consolidation of lung parenchyma--Only the saline/saline control group differed from the other groups in lung consolidation; the three paraquat-exposed groups did not differ from one another. Hence, L-DHP and DL-DHP did not reduce the pulmonary fibrosis resulting from paraquat exposure.

Relative numbers of pulmonary epithelial cell types (types I and II pneumocytes)--Although some fluctuations in type I and type II pneumocytes numbers occured within and throughout the various groups,

no statistically significant differences were seen, and no consistent trends were noted due to various treatments. However, a trend was present of decreased number of type I pneumocytes and increased number of type II pneumocytes in the paraquat/saline group versus the L-DHP-treated group (e.g., an increase from 23.3 to 26.7 during day 10 to 18 in the paraquat/L-DHP group).

Comparison of the paraquat/DL-DHP and paraquat/saline groups from day 39 to day 100 showed no significant differences between each at day 39. However, on day 100 the number of macrophages, the number of pulmonary cells and the alveolar wall thickness of the paraquat/DL-DHP group were statistically significantly lower than those of the paraquat/saline group (Table 2).

Discussion

The proline analogs, L-DHP and DL-DHP, which competitively inhibit the incorporation of proline into collagen[7,13] were used in rats to test their potency as treatments for paraquat poisoning. Since collagen is high in proline content, the replacement of proline by L- or DL-DHP will affect collagen metabolism[7,13] by preventing the aminoacylation of t-RNA by proline, by the reduction of hydroxylation of prolyl residues, and by the reduction of collagen secretion from the fibroblasts.[7] However, the inhibitory efficiency of these analogs had not been demonstrated in vivo.

In the present study, there was no consistent statistically significant benefit on the pulmonary effects of paraquat toxicity by L-DHP and DL-DHP treatments when they were administered 10 days after

the initial paraquat exposure. Of the several parameters measured, only the number of intra-alveloar macrophages and the total number of pulmonary cells had a significant decrease after L-DHP or DL-DHP treatment when compared to the paraquat/saline group. This correlation is consistent with observations that paraquat stimulates fibroblasts and that it achieves this through the medium of macrophages,[14] although the specific nature of this stimulation is unknown. The origin of infiltrating intra-alveolar profibroblasts (primitive mesenchymal cells which mature into fibroblasts) is unknown, but it has been suggested that the profibroblasts derive from primitive histiocyte-like cells which are then transformed into fibroblasts.[8] The finding that L-DHP significantly reduced the number of intra-alveolar macrophages supports the beneficial use of these proline analogs in treating this aspect of in vivo paraquat intoxication.

The absence of significant differences in the number of leucocytes/fibroblasts within and among the various groups suggests failure of L-DHP or DL-DHP to stop the proliferation of fibroblasts and the synthesis of collagen in lung parenchyma during paraquat intoxication. The number of macrophages was reduced significantly ($p<.05$) by the L-DHP treatment, but no differences were observed in the degree of lung consolidation or mean alveolar wall thickening of the 3-paraquat-dosed groups. This apparent lack of effect by L-DHP or DL-DHP on the collagen-synthesizing fibroblasts is at variance with the observation citing macrophages as the medium for fibroblast stimulation during paraquat poisoning.[14]

Dehydroproline compounds minimized the destruction of type I pneumocytes and the hyperplasia (increase) of type II pneumocytes. Some investigators[8,15] believe these are the basic pathological changes in paraquat poisoning. On this basis, L-DHP gave some indication of having reduced the degree of experimentally-induced paraquat lung toxicity. The lesser effect of systemically administered paraquat versus that of exposure to paraquat aerosol,[16] may account for the minimal pneumocyte effect observed following the subcutaneous paraquat exposure of this report.

The lack of any significant difference, except marginal changes on day 100 in macrophage number, number of pulmonary cells and alveolar wall thickness in the DL-DHP treated group (Table 2), suggests that pulmonary effects due to paraquat are initiated early in the syndrome. These effects will only minimally improve with treatment over time, once the changes are present. The observations after day 39 were largely consistent with earlier observations before day 39. However, the data presented here suggest that perhaps the response may have been better had L-DHP been started within 1-4 days of paraquat exposure instead of after 11 days post-paraquat exposure. The potential benefit of DHP treatment should not be discarded until studies using more concurrent paraquat exposure and DHP treatment are carried out.

Under the conditions of this present study, there was insufficient evidence to show that L-DHP or DL-DHP significantly decreased the pulmonary fibrosis of experimental paraquat poisoning. However, the observations do not rule out the possible value of DHP treatment if

given earlier in the paraquat toxicity syndrome. Further work needs to be done to determine the pathogenesis of paraquat intoxication, since pulmonary fibrosis and consolidation progressed in the face of DHP therapy rather than diminishing as one would anticipate given the stated mechanism of DHP on collagen synthesis.

FIGURE CAPTIONS

Fig 1--Structural formula for paraquat dichloride

Fig 2--Experimental design for the study of the effect of dehydroproline (L-DHP or DL-DHP) on the response of rat lungs to paraquat exposure. Groups are designated saline/saline, paraquat/L-DHP, paraquat/DL-DHP and paraquat/saline.

Fig 3--Lung section from rat that received saline injection for 8 days (Saline/Saline control group). Day 10. Alveolar walls normal with little cellular infiltrates. X400.

Fig 4--Lung section from rat that received 7 mg paraquat/kg body weight subcutaneously for 8 days (Paraquat/L-DHP). Day 10. Alveolar wall thickness increased while number of the intra-alveolar leucocytes/fibroblasts is decreased. X370.

Fig 5--Lung section from rat that received 7 mg paraquat/kg body weight subcutaneously for 8 days (Paraquat/DL-DHP. Day 10. Alveolar walls markedly increased in thickness. Infiltrating leucocytes/fibroblasts are significantly decreased in number/0.08 mm^2 of lung sample. X400.

Fig 6--Lung section from rat that received 7 mg paraquat/kg body weight subcutaneously for 8 days followed by 25 mg DL-DHP/kg body weight for 14 days (Paraquat/DL-DHP group). Day 25. Marked increase in the alveolar wall thickness and in consolidation. X350.

Fig 7--Lung section from rat that received 7 mg paraquat/kg body weight subcutaneously for 8 days followed by daily saline injection for 14 days (Paraquat/Saline positive control group). Day 39.

Collapse of lung alveoli, thickening of the alveolar walls, increased consolidation of the lung parenchyma, and marked alveolar cellular infiltration by leucocytes/fibroblasts. X350.

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Table 1: The mean ± standard error values of the various parameters under consideration for the groups of experimental rats taken on days 10,18,25,32,39, and 100 after dosing 7-8 days with saline and then treated for 14 days (day 11-24) with saline (saline/saline group), or after dosing 7-8 days with paraquat (PQT) and then treated for 14 days (day 11-24) with L-DHP (PQT/L-DHP group), DL-DHP (PQT/DL-DHP group) or saline (PQT/saline group).

Group	Day of Sacrifice After Dosing Was Started	No. of Animals	*No. of Macrophages	*No. of Leucocytes/ Fibroblasts	*Total No. of Pulmonary Cells	*Alveolar Wall Thickness (um)	Consolidation of Lung Parenchyma (%)	*Relative No. of Type I Pneumocytes (%)	*Relative No. of Type II Pneumocytes (%)
Saline/Saline	10	4	21.2±1.37	2.8±0.74	24.0±1.61	2.3±0.17	1.0±4.82	70.8±4.08	29.2±4.12
	18	4	20.2±1.37	2.2±0.74	22.5±1.61	2.5±0.17	1.2±4.82+	76.8±4.08	23.2±4.12
	25	4	20.0±1.37	3.2±0.74	23.2±1.61	2.4±0.17	1.2±4.82+	72.2±4.71	27.8±4.75
	32	4	21.2±1.37	4.0±0.74	25.2±1.61	2.5±0.17	1.0±4.82+	78.4±4.08	21.6±4.12
	39	4	17.8±1.37	4.5±0.74	22.0±1.61	2.5±0.17	1.0±4.82+	84.5±4.08	15.5±4.12
PQT/L-DHP	10	4	23.6±1.23	6.0±0.66	29.6±1.44	3.7±0.17	25.8±4.82	76.7±4.71	23.3±4.75
	18	4	14.5±1.37+	3.5±0.74+	18.0±1.61+	3.6±0.17	17.5±4.82	73.3±4.08	26.7±4.12+
	25	4	11.0±1.37+	3.0±0.74+	14.0±1.61+	3.9±0.17	21.5±4.82	75.8±4.08	24.2±4.12
	32	4	11.5±1.37+	2.2±0.74+	13.8±1.61+	3.8±0.17	23.0±4.82	76.1±4.08+	23.9±4.12
	39	4	15.0±1.37+	2.5±0.74+	18.5±1.61+	4.2±0.17	19.2±4.82	72.6±4.08	27.4±4.12+
PQT/DL-DHP	10	4	10.7±1.59	2.3±0.85	13.0±1.86	3.5±0.17	13.5±4.82	57.1±8.15	42.9±8.23
	18	4	11.8±1.37	2.0±0.74	13.8±1.61	3.8±0.17	18.5±4.82	79.2±4.08	20.8±4.12
	25	4	11.5±1.37	2.2±0.74	13.8±1.61	3.8±0.17	21.8±4.82	79.0±4.08	21.0±4.12
	32	3	10.3±1.59	2.3±0.85	12.8±1.86	3.9±0.20	22.7±5/57	77.3±4.71	22.2±4.75
	39	4	10.0±1.37	3.2±0.74	13.2±1.61	3.8±0.17	23.2±4.82	70.8±4.08	29.2±4.12
	39	3	13.0±1.37	2.0±0.74	15.0±1.61	4.3±0.17	26.0±4.82	63.4±7.32	36.6±5.57
	100	4	10.0±1.37	2.0±0.74	12.0±1.33	4.5±0.21	32.5±4.40	63.2±7.32	38.8±7.15
PQT/Saline	10	3	11.0±1.59	2.3±0.85	13.3±1.86	3.7±0.25	17.7±5.57	69.0±5.77	31.0±5.82
	18	4	21.5±1.37	1.5±0.74	23.0±1.61+	3.8±0.17	22.8±4.82	70.6±4.08	29.4±4.12
	25	4	19.8±1.37	2.0±0.74	21.8±1.61+	4.0±0.17	24.8±4.82	67.5±4.08	32.5±4.12
	32	4	18.8±1.37	2.8±0.74	21.5±1.61+	4.1±0.17	25.8±4.82	67.9±4.08	31.6±4.12
	39	4	21.2±1.37	2.5±0.74	23.0±1.61+	4.1±0.17	27.5±4.82	67.7±4.08	33.8±4.12
	39	3	17.5±1.37	3.2±0.74	20.7±1.61	4.2±0.17	25.0±4.82	65.5±3.58	35.5±4.64
	100	4	17.2±1.37	2.8±0.74	20.0±1.91	4.8±0.17	35.0±4.04	61.2±3.58	38.8±6.67

*Area of field of view for these parameters is 0.08 mm^2, excepting the consolidation of lung parenchyma and relative number of cell types (type I and type II pneumocytes), which are expressed in percent of total, all the other parameters are in micrometer (um).
+Value is statistically different at p 0.05 when compared to the day 10 value within the same group.

Table 2: Analysis of variance of paraquat (PQT)/DL-3,4-dehydroproline (DL-DHP) and PQT/saline groups of rats sacrificed on days 39 and 100 after dosing was started.

	Paraquat/DL-DHP Group					Paraquat/Saline Group				
	Day 39	Day 100	Critical Values for Comparing Days*	Day 39-100 Value Differences PQT/DL-DHP	PQT/Saline	Day 39	Day 100	Critical Values for Comparing Days*	Day 39-Day 100 Value Differences PQT/DL-DHP	PQT/Saline
No. of Macrophages	13	10	3.81	3.9+	0.258	17.5	17.2	4.66	4.50	7.25+
No. of Leuco-cytes/Fibro-blasts	2	2	2.04	0.0	0.50	3.2	2.8	2.51	1.25	0.75
Total Number of Pulmonary Cells	15	12	4.46	3.0	0.75	20.7	20.0	5.47	7.75+	8.00+
Alveolar Wall Thickness (um)	4.3	4.5	0.48	0.18	0.65+	4.2	4.8	0.59	0.12	0.35
Consolidation of Lung Parenchyma (%)	26	32.5	13.36	6.5	10.0	25	35	16.36	1.00	2.50
Type I Pneumocyte (%)	63.4	62.2	11.31	1.35	4.3	65.5	61.2	13.84	2.15	0.80
Type II Pneumocyte (%)	36.6	38.8	11.41	1.35	4.3	34.5	38.8	13.98	2.15	0.80

*Values above which the group difference must exceed to be statistically significant.
+Statistically significant at p 0.05.

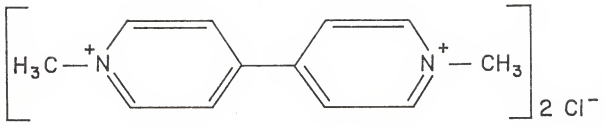

Fig 1.

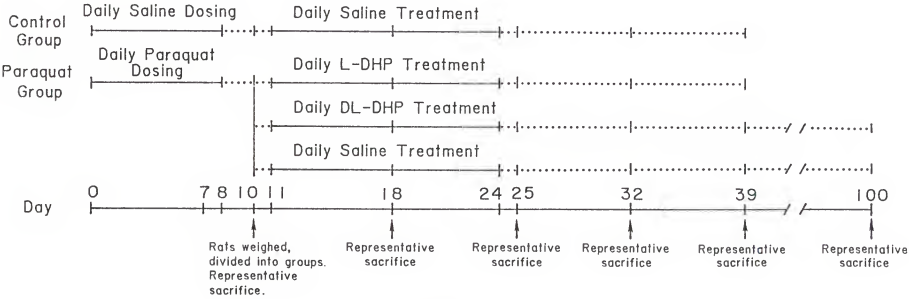
Control Group
Daily Saline Dosing
Daily Saline Treatment
Paraquat Group
Daily Paraquat Dosing
Daily L-DHP Treatment
Daily DL-DHP Treatment
Daily Saline Treatment
Day
0
7 8 10 11
18
24 25
32
39
100
Rats weighed, divided into groups. Representative sacrifice.
Representative sacrifice
Representative sacrifice
Representative sacrifice
Representative sacrifice
Representative sacrifice

Fig 2.

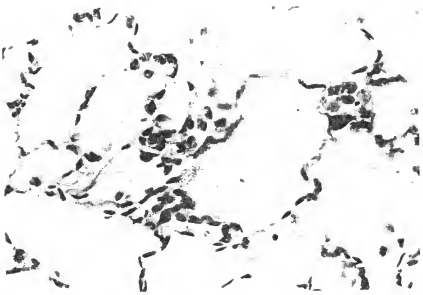

Fig 3.

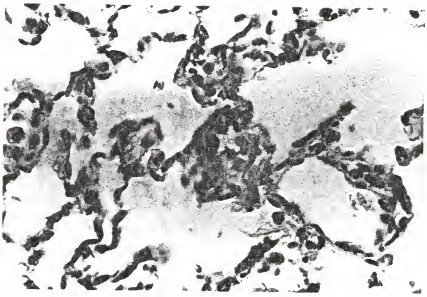

Fig 4.

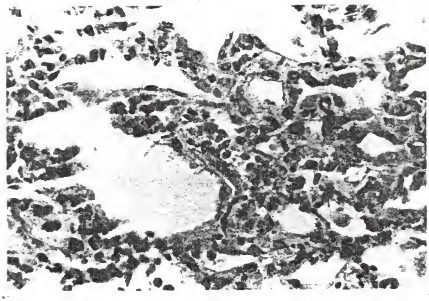

Fig.5.

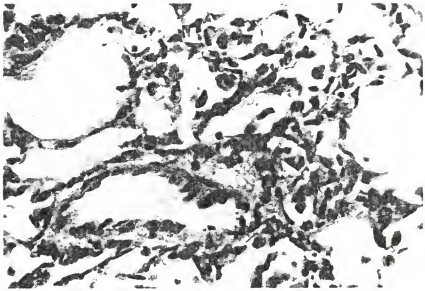

Fig 6.

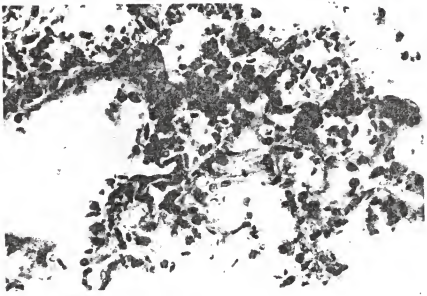

Fig 7.

APPENDIX I

A LITERATURE REVIEW OF PARAQUAT AND RELATED CHEMICAL TOXICITIES

CLINICAL SIGNS OF PARAQUAT POISONING

The initial signs of paraquat poisoning depend on the amount of paraquat ingested. Ingestion of a massive dose (ie, more than 6 oz) of the concentrated paraquat results in severe local irritation of the mouth, pharynx, esophagus and stomach, accompanied by nausea, profuse repeated vomition, and often abdominal cramps and diarrhea. Usually this is accompanied by severe multisystem injury with total renal shutdown and centrilobular necrosis of the liver. The victim may become semi-comatose within a few hours of the ingestion; fatal pulmonary edema may develop and death supervenes in a few days.

Ingestion of a lesser but potentially fatal amount (ie, less than 0.5 oz) of the concentrated paraquat leads to nausea in the victim. Human victims may complain of a sore mouth and throat the day after ingestion. The proper diagnosis may be missed altogether if a history of paraquat ingestion is not available. By the second or third day there may be clinical evidence of kidney and liver injury with decreased urinary output and jaundice. Laboratory tests reveal rising blood urea nitrogen and creatinine, and elevated serum transaminase and bilirubin levels. With adequate treatment the kidney and liver changes are reversible and non-fatal. However, the possibility of death may be difficult for both the victim and the medical personnel to accept, since clinical signs of impending respiratory failure may be few or absent. Nevertheless, pulmonary function tests are likely to be abnormal and changes may also be visible in chest radiographs (increased density of the lungs). Renal and hepatic function may return to normal, but the victim develops increasing respiratory

distress caused by progressive pulmonary fibrosis. Death ensues within several days or weeks (1).

PATHOGENESIS OF PARAQUAT-INDUCED PULMONARY FIBROSIS

Following ingestion and absorption of paraquat from the gastrointestinal tract, there is a heavy infiltration into the pulmonary alveolar spaces of primitive connective tissue cells or profibroblasts. These profibroblasts undergo maturation into large basophilic fibroblasts which occlude many alveolar spaces. Later, the alveolar walls disintegrate, permitting the individual clumps of fibroblastic tissue to coalesce, resulting in obliteration of the lung alveolar architecture. The fibroblasts lay down a small quantity of collagen, but death from respiratory failure usually occurs before this has become extensive. This final stage of the disease (deposition of collagen), is reminiscent of interstitial fibrosis of the lung, but obliteration of the alveolar walls bellies its true intra-alveolar origin.

It has been suggested that paraquat causes interstitial pulmonary fibrosis simply by organization of inflammatory exudate (2). Incorporation of organizing inflammatory exudate or cellular debris into the alveolar walls can lead to interstitial fibrosis (3). However, chronic administration of paraquat to rats induces an extensive fibrosis following only a slight fibrinous exudate (4,5). It has also been shown that inhalation of paraquat aerosols by animals causes an extensive exudation of fluid into the alveoli, but this does not result in pulmonary fibrosis (6). It seems that the formation of a fluid

exudate during the early stages of paraquat poisoning is not necessary for the development of pulmonary fibrosis and that paraquat, or its metabolities, directly stimulate the infiltration of profibroblasts into the alveoli of the lungs. The experimental evidence from rats, plus reported cases in man and domestic animals, suggests that the pathogenesis of the paraquat lung is similar in all species. The basic pathological change in the human and animal lung from paraquat poisoning is a diffuse cellular, intra-alveolar fibrosis and not a fibrosing alveolitis.

Caution must therefore be exercised in comparing the pathology of experiments in control animals with those in human and domestic animal disease, since those of human and domestic animals are often modified by treatments such as oxygen therapy. In human cases in which 40% oxygen had been used, there was an early exudative phase characterized by hyaline membranes, followed by a proliferative phase in which there was a hyperplasia of granular pneumocytes and the development of interstitial pulmonary fibrosis (7). In some cases (8) there was also an intra-alveolar fibrosis. There is no doubt that paraquat is capable of producing pulmonary fibrosis on its own, for intra-alveolar fibrosis occurs when paraquat is given to rats breathing only atmospheric oxygen; but one should consider the possibility that oxygen toxicity may be a contributory factor in the pathogenesis of the pulmonary fibrosis in some cases of paraquat poisoning in man and animals. Furthermore, the histopathology of paraquat and oxygen poisoning is somewhat different in that, although intra-alveolar fibrosis may occur in the latter, there is always a prominent interstitial component in

the lungs (7). Further, there is no profuse hyperplasia of granular pneumocytes in paraquat poisoning since most alveolar walls disintegrate and become engulfed by fibroblastic tissue.

SYNERGISM OF OXYGEN AND PARAQUAT IN PARAQUAT POISONING

Hypoxic treatment has been advocated in paraquat poisoning, since oxygen and paraquat have a synergistic effect in producing lung injury (9,10). In two of three cases of fatal paraquat poisoning reported (11), victims were given oxygen in the course of their hospitalization. In the first of these two cases, hypoxic treatment was given until life could no longer be sustained without a high concentration of oxygen. In the second case, paraquat poisoning was not suspected and the victim received oxygen after the drop in arterial pO_2. In both cases, it is possible that the use of oxygen contributed in some degree to the formation of the hyaline membranes and fibrosis seen histologically. The victim in the third case did not receive oxygen, yet there was beginning hyaline membrane formation. Except for superoxide dismutase and immunosuppressive therapy, all other accepted therapeutic modalities were used in these three lethal cases without positive results.

PATHOLOGIC FINDINGS IN PARAQUAT POISONING IN HUMAN AND EXPERIMENTAL CASES

Paraquat is a potent multisystem poison for which no known antidote has been discovered. The clinical effects of paraquat poisoning seem to be dose-related. There is acute simultaneous multisystem failure with fatalities in a few days following ingestion of a large dose. In smaller doses there is less severe, often transient involvement of

the liver, kidneys, gastro-intestinal tract and progressively worsening, irreversible pulmonary fibrosis which leads to respiratory insufficiency and death within a few weeks following ingestion.

The following pathologic findings were observed in three fatal human cases of paraquat poisoning (11). In case 1, a 28-year-old male orchard worker intentionally ingested 2.5 cups of 21% paraquat solution, 40 Tylenol tablets, 10 asprin tablets and 20 pro-banthine tablets. Jaundice, central and peripheral congestion, and edema were noted at autopsy. There was congestion of the respiratory tree and both the trachea and bronchi contained bloddy liquid. The lungs were heavy, airless, markedly congested and edematous with focal areas of hemorrhages. The liver was soft and yellow. The mucosa and wall of the stomach were edematous. The myocardium appeared light brown and mottled. Histological examinations showed toxic myocarditis, congestion, edema with focal hemorrhages, atelectasis and focal hyaline membrane formation in the lungs. There was centrilobular necrosis of the liver, and the kidneys had acute tubular necrosis. The adrenals contained cortical necrosis with focal hemorrhages.

Case 2 followed intentional ingestion of 20% paraquat solution. Treatment was started 18 hours after the ingestion. Post mortem findings included intense icterus of the skin. The lungs were heavy, airless, congested, edematous and had considerable fibrosis. The bronchi contained blood-tinged mucous. There was marked enlargement and greenish discoloration of the liver, and a section through it showed a nutmeg pattern. The kidneys were also enlarged with marked cortical congestion. Histopathological examination of the lungs revealed congestion, edema, focal hemorrhages, marked intra-alveolar

and interstitial pulmonary, fibrosis with a honeycomb appearance, and foci of epithelial hyperplasia. The liver had congestion and centrilobular necrosis, focal erosive damage of the epithelium of the bile ducts and mild periportal inflammation. There was acute tubular necrosis of the kidneys with some regeneration.

In case 3, a 57-year-old male was admitted to the hospital after several days of nausea, diarrhea, glossitis and pharyngitis with a diagnosis of probably viral syndrome. X-rays revealed a worsening pneumonitis. Paraquat poisoning was not suspected until shortly before the patient's demise. Autopsy findings were heavy and airless lungs. The pleural surfaces contained areas of fibrinous exudate and extensive fibrous adhesions. There were also foci of pneumonia with mircoabscess formation. Trachea and bronchi contained small amounts of bloody mucoid material. The liver was greasy and enlarged. There was cardiomegaly, and the cerebral hemispheres contained numerous petechial hemorrhages within the white matter. The esophagus, stomach and larynx had superficial focal erosions. Histological examination revealed severe intra-alveolar and interstitital pulmonary fibrosis with honeycombing, marked epithelial hyperplasia and squamous metaplasia of the bronchial epithelium, pneumonia and abscess formation. There was acute renal tubular necrosis with regeneration. Petechial hemorrhages of the brain were also observed, but the liver had minimal fatty change. Myocardial necrosis, adrenal cortical necrosis and focal esophageal, gastric and laryngeal erosions were minimal.

These cases illustrate the fatal outcome of large doses of paraquat poisoning and the multisystem effects. The liver, kidneys, adrenals and brain may become affected, but the fatal effect of paraquat

poisoning tends to arise from respiratory insufficiency as a result of intra-alveolar and interstitial fibrosis of the lungs. The cholangiocellular injury involving small and medium sized bile ducts has been reported after paraquat ingestion (13). Paraquat is excreted into the bile largely unchanged, and a possible reason for the bile duct injury may be the local irritating effect of the compound (13). As a result of biliary excretion of paraquat in a largely unchanged state, bile may be successfully used to confirm the presence of paraquat.

Observations in experimental studies of paraquat poisoning using rats (5,14) showed that large doses of paraquat administered to rats caused central nervous derangements, particularly of locomotor activity. The choking respiration with which these signs were associated may also be the result of central neuropathy. However, the dyspnea which developed was attributed to extensive alveolar edema following acute paraquat intoxication. At this state of the disease, death was presumably ascribed to the combined effects of central neuropathy and acute respiratory failure.

Pulmonary fibrosis, which developed in rats after treatment with paraquat, was demonstrated to originate from an infiltration into the alveolar spaces of primitive mesenchymal cells, profibroblasts (5,14). In an acute experiment, in which doses of up to 50-100 mg paraquat/kg were used, dense cellular fibrosis with obliteration of alveolar spaces was demonstrated. Electron microscopy showed this fibrosis to be exclusively intra-alveolar in nature. The damage to the alveolar walls was so severe that many were disruptured with just the cappillaries remaining intact. In the chronic experiments, the sequence of changes leading to the maturation of profibroblasts into mature

mature fibroblasts was traced. There was an initial loss of pseudopodia of the profibroblasts and an increase in number and size of mitochrondria. The rough endoplasmic reticulum became more extensive. All of these changes resulted in the production of intermediate forms, the elongation of which formed mature fibroblasts. Maturation changes observed at the light microscopic level comprised a loss of the "ragged" outline of primitive mesenchymal cells with an increase in size and basophilia of the cytoplasm.

The development of this intra-alveolar fibrosis in the chronic administration of paraquat was extremely rapid and death occurred 8 to 10 days after the onset of clinical signs. This rapid development was explained to be due to a large number of infiltrating profibroblasts and the persistent supply of oxygen and nutrients to the developing fibrous tissue (5). Although the reason for the formation of the fibrosis in the first instance is not clear, it was suggested that it arose simply by fibrous repair of damaged alveolar walls (2). But this did not explain the origin of the intra-alveolar fibrosis. Also, the destruction of the alveolar epithelium by chronic oxygen poisoning produced only mild focal scarring and not the classic florid fibrosis typical of paraquat poisoning in man (15). The fibrosis did not appear to develop from organization of an acute inflammatory exudate since in a chronic experiment the inflammatory reaction was mild and the alveoli contained little fribin. Additionally, inhalation of paraquat aerosols produce more damage to the alveolar epithelium than did systemic administration but pulmonary fibrosis never resulted (6). It was reported that Conning and

co-workers, in 1969, observed that paraquat actively stimulated fibroblasts and that it achieved this through the medium of macrophages (14). The fact that paraquat produced pulmonary fibrosis only when administered systematically, and that pulmonary fibrosis usually developed after paraquat concentration in the body had dropped below detectable levels, suggests that it is first metabolized in another organ such as the liver (16). It appears that the acute phase of paraquat poisoning, consisting of pulmonary edema and disintegration, is not the necessary prerequisite for the development of subsequent pulmonary fibrosis. In an experimental study, pulmonary fibrosis developed from an intra-alveolar infiltration of pro-fibroblasts, but where these cells come from still remains unknown (5). In the early stages of chronic paraquat poisoning, many primitive mesenchymal cells were observed in the vicinity of the bronchioles and blood vessels. This observation was also described as an interstitial infiltrate of primitive histiocyte-like cells which appeared to transform into fibroblasts (2). It was suggested that these histiocytes originated from blood monocytes; however, no evidence of interstitial proliferation or migration of histiocytes (ie, macrophages) was revealed (5,14).

Investigation of the pathogenesis and structure of the paraquat-exposed lung in rats found the presence of muscularized pulmonary arterioles, suggesting that pulmonary hypertension had existed during life (5). These hypertensive pulmonary arterioles were observed in a chronic experiment in some rats with the most extensive pulmonary fibrosis. It was theorized that these rats developed hypoxic hypertensive pulmonary vascular disease secondary to fibrosis, in like manner

as occurs in many human forms of pulmonary fibrosis (5).

In some of the rats in the chronic experiment foci of compact interstitial fibrosis, patent alveoli, alveolar macrophages and a continuous epithelial lining of granular pneumocytes existed. Why such a lestion developed was unclear, although an identical histological picture has been described in parts of the lungs of rats after prolonged intoxication with Crotalaria spectabilis seeds (4). The significance of these foci of pulmonary changes is not known but it is unlikely that they play a major role in the paraquat-exposed lung.

TREATMENT AND MANAGEMENT OF PARAQUAT POISONING

The best course of management and treatment of cases of paraquat poisoning is still debatable. Gastric lavage using Bentonite or Fuller's earth has been tried to prevent further absorption of the compound (17). Various techniques designed to eliminate paraquat from the tissues include forced diuresis, hemodialysis, charcoal hemoperfusion, and plasmaphoresis (18). None of the elimination techniques are very effective. Some reports say that less than 2% of the estimated absorbed dose can be removed by hemodialysis. Charcoal hemoperfusion has been reported to be approximately five times more effective than hemodialysis (19). Oxygen therapy has been reported to worsen the paraquat-induced lung injury, thus hypo-oxygen treatment has been advocated (10). Based on experimental data, treatment with superoxide dismutase (SOD) has been advocated, since SOD is known to remove the superoxide radical O_2^-, which has been proposed as the toxic intermediary of paraquat poisoning (20).

Immunosuppressive therapy has also been attempted (21). None of these techniques is without some hazard to the patient, and the success rate is, at best, modest.

Recent attempts at treatment of paraquat poisoning involved surgical lung transplant in combination with injectable cyclosporine over a long period of time to minimize the possibilities of tissue rejection. There are attempts also in using inhibitors of collagen synthesis, e.g., 3,4-DL-dehydroproline and 3,4-L-dehydroproline (22), to treat paraquat poisoning in rats.

Modalities in the attempts to treat paraquat poisoning in man and animals include the following:

(i) Use of antioxidants--Natural vit E and synthetic butyl-hydroxyl toluene (BHT)

(ii) Use of clofibrate (ethyl-p-chlorophenoxyisobutyrate; atromid-s)

(iii) Use of superoxide dismutase (SOD)

(iv) Hypoxic protection

(v) Use of N-acetyl-L-cysteine (NAC)

(vi) Use of niacin

Mechanism of Paraquat Poisoning and Vitamin E Therapy

Vitamin E. Since it has been established that paraquat functions as a redox agent and contributes to the formation of oxygen free radicals, many experiments have been conducted to explore mechanisms which might reinforce their formation, accelerate their degradation to less toxic products, and interrupt the chain of reaction of lipid peroxidation

through the use of antioxidants or free-radical scavengers.

The possible mechanisms of paraquat toxicity in mammals and how they relate to therapy directed against the superoxide radical shows that in the presence of reduced nicotinamide adenine dinucleotide phosphate (NADPH) (Fig 1), paraquat can be reduced in a one-electron transfer process by microsomal NADPH-cytochrome reductase. Paraquat oxidation is coupled with the reduction of molecular oxygen resulting in the formation of superoxide anion, singlet oxygen and possibly hydroxyl radicals. These molecular species can react with polyunsaturated fatty acids forming fatty acid free radicals and, with further oxidation, lipid hydroperoxide radicals. The hydroperoxide radicals then maintain the formation of new fatty acid radicals while being converted to lipid peroxides in a chain reaction. Lipid peroxidation is destructive to cell structure and function by damaging mitochondria, microsomal organelles and other elements. The measuring of malondialdehyde formation has become a conventional procedure for monitoring lipid peroxidation and hence confirming that paraquat causes lipid peroxidation (23). Several enzymes are present in the cells that catabolize the superoxide radical and reduce the lipid hydroperoxides to less toxic lipid alcohols. The superoxide anions are converted to H_2O_2 and O_2 by SOD, and the H_2O_2 is further inactivated to H_2O and O_2 by the enzyme, catalase. The reduction of lipid peroxides by glutathione peroxidase requires reduced glutathione (GSH). Since the reduction of oxidized glutathione is coupled with the oxidation of NADPH through glutathione reductase, it does seem evidently that the availability of sufficient NADPH is a critical factor for the detoxification process (24).

Exposure of rats to high oxygen concentrations increases paraquat mortality (9). In other experiments, low oxygen mixtures were shown to reduce paraquat mortality and have lead to guidelines to limit oxygen in poisoned patients. But because of the need for maintenance of adequate peripheral oxygenation in the severaly affected patient showing respiratory distress overrides these principles, oxygen is usually administered. Although experimental studies using SOD seemed to indicate that administration of the enzyme has protective effects, clinical use of SOD has been disappointing (25). It is believed that the inability of the protein molecule to penetrate cells, and its rapid inactivation might be factors explaining its lack of significant effect.

Since vitamin E is an antioxidant and hence acts as reducing substance and free-radical scavenger, it is rationalized that vitamin E can quench superoxide radicals (26), and lipid-free radicals, thus interrupting the chain reaction. Experimental studies demonstrated greater paraquat mortality in mice maintained on a vitamin E deficient diet than those receiving a vitamin E supplemented diet (27,28). The possibility existed that vitamin E treatment, while not effective in decreasing the acute death rate, could reduce the development of pulmonary fibrosis in paraquat poisoning (24). The lack of an acute beneficial effect of vitamin E further suggests that cellular NADPH depletion rather than free radical mechanisms determine paraquat toxicity.

Butyl-hydroxyl toluene (BHT). Paraquat appears to exert a toxic activity only in the presence of oxygen. The free radical $O_2^{\cdot -}$ compounds initiate an oxidative reaction in cell membranes and membranes of the cytoplasmic organelles, so-called lipid peroxidation. This action takes place especially in the lipid midzone of the membrane containing chains of multiple unsaturated fatty acid (29).

If indeed paraquat poisoning is based on lipid peroxidation (30), lipid-soluble antioxidants should be helpful in preventing signs of intoxication. Consequently, the lipophilic synthetic antioxidant, BHT, was tried as a potential antidote for paraquat poisoning (30), and the following observations were made. The lipophilic antioxicant BHT diminished mortality in rats intoxicated with paraquat. Post-mortem histological examination also showed a favorable influence of BHT on lung lesions. These findings support the hypotehsis that in paraquat intoxication lipid peroxidation occurs and may be responsible for the lung lesions. Whether BHT could be used as a therapy in human and animal intoxications is still questionable in view of the observed BHT toxicity in experiments where rats were pretreated with BHT before the administration of paraquat (30).

Clofibrate

Clofibrate is used clinically as a preventative against premature atherosclerosis and coronary heart disease (31). As a result of reported increased hepatic antioxidant enzyme catalase activity following clifibrate treatment (32), clofibrate was tested for its effect on lung antioxidant enzyme activity, and concurrently, as a possible protective agent

for the paraquat-exposed lung (33). Since clofibrate could enhance lung catalase and other antioxidant activity, it might have a protective action against paraquat-induced lung toxicity.

The clofibrate solution was administered either by subcutaneous injection or by oral feeding with stomach tube in a dose of 0.5 ml/100 g body weight. Clofibrate was diluted to a concentration of 12 mg/ml and preadministered before the dose of 35 mg paraquat/kg was given. Only 28% and 29% of the untreated controls were alive 10 days post-paraquat as opposed to 70% of the clofibrate-pretreated animals (33). The paraquat-exposed rats had extensive perivascular edema and localized areas of intra-alveolar edema and/or hemorrhages, whereas the rats pretreated with clofibrate showed minimal or no perivascular fluid accumulations and no areas of intra-alveolar edema or hemorrhages. No significant changes in catalase activity or any other potentially protective lung enzymes were found. It appeared that clofibrate's protective action against paraquat was not due to its augmentation of these lung defenses against $O_2^{\cdot -}$ free radical (paraquat) injury.

In other experiments in which clofibrate was administered after the lethal dose of paraquat (instead of the pretreatment regimen), the 10-day survival rate was no different in the clofibrate-treated animals versus the paraquat control rats. While it is unfortunate that clofibrate administration after paraquat was not protective against paraquat-induced lung toxicity, if the protective mechanism of the pretreatment regimen can be unraveled, it may provide valuable clues to suggest effective post-paraquat treatment approaches (33).

Superoxide Dismutase (SOD)

Paraquat has a low reduction potential and if reduced can utilize molecular oxygen as a one-electron acceptor (34). Diquat (1,1'-ehtylene-2,2'dipyridylium dichloride), an analogue of paraquat was found to produce superoxide free radicals ($O_2^{\cdot -}$) when reduced aerobically by NADPH and glutathione reductase or photochemically in the presence of EDTA (25). The production of $O_2^{\cdot -}$ free radicals was inhibited by SOD, an enzyme existing in all oxygen-utilizing cells and which is known to remove $O_2^{\cdot -} + 2H^+ \longrightarrow H_2O_2$; SOD is thought to be the primary defense against the potential damage to cells from free-radical species. These considerations suggest that the administration of SOD to animals intoxicated with paraquat might exert a protective effect against paraquat toxicity.

Several experiments conducted in this field (25) revealed the following: (i) There was a delay of several hours before death supervened in paraquat-treated rats treated with SOD when compared to paraquat-treated rats which did not receive SOD; (ii) Although areas of hypercullularity were observed after treatment with paraquat and SOD, the areas of cellular infiltration were much more reduced and the retention of extensive areas of normal alveolar structure much greater when compared with the lungs of those animals which did not receive the enzyme treatment.

By the method of grid-overlay, in which unit areas of normal alveolar structure and of hypercellularity or inflammation were counted and compared, analysis showed the percentage of abnormal alveolar structure within the total field of the lung section from the paraquat-treated rat was almost three times that of the controls.

The value obtained upon similar analysis of the lung tissue from the SOD and paraquat-treated animals was one-half of the value obtained from the animals treated with paraquat along (25):

Rat Treatment	Abnormal Alveolar Structure (%)
Air	24
Air + Paraquat	70
Air + Paraquat + SOD	35

SOD extended by more than two-fold the lifetime of animals treated with paraquat and maintained in air. SOD also reduced the microscopically-observable damage in lung tissue of these rats. Such modest response of the paraquat-exposed lung in rats to SOD therapy (25) may be useful in combination with other therapeutic procedures in the treatment of pulmonary fibrosis caused by paraquat poisoning in man and domestic animals.

Hypoxic Protection

Since its introduction as a herbicide in 1962, paraquat has caused the death of more than 120 individuals. Fatalities were due to accidental or intentional ingestion, rather than by inhaling the aerosol (6). But regardless of the route of administration, the lungs are the major site of pathologic changes caused by paraquat.

Paraquat toxicity in both plants and animals is believed to be mediated by the ability of this chemical to accept electrons from cell components, eg, flavo-proteins or chlorophyll, and to transfer these

electrons directly to molecular oxygen. This single-electron transfer leads to formation of superoxide radicals which peroxidize membrane lipids and enzymes, thus disrupting normal metabolic pathways (10). Investigators (35), quoting their earlier work, believed that the superoxide radical may play an important role in the pathogenesis of oxygen toxicity. Another work (9) pointed out that the pulmonary pathological changes induced by paraquat in many ways resembled those of oxygen toxicity. This study reported that Escherichia coli grown aerobically was inhibited by paraquat, whereas E. coli grown anaerobically was not affected by paraquat, indicating the importance of the ambient oxygen concentration on paraquat toxicity (9).

Due to reports of accelerated paraquat toxicity in elevated oxygen concentration, experimental studies showed a protective effect of hypoxia on paraquat toxicity, and a decreased cumulative mortality rate of mice parenterally injected with paraquat (10). This protective effect of hypoxia may simply be due to lowering of the concentration of one of the constituents of the toxic chemical reaction. The lungs and in particular the alveolar epithelia are the primary target (10) for the following reasons:

(i) The alveolar pO_2 was higher than in any other part of the body; and

(ii) Paraquat was concentrated in the lung.

Paraquat poisoning is not the only form of pulmonary insult aggravated by increased levels of oxygen. Viral and pneumonococcal infection, nitrogen dioxide exposure, and intravenous oleic acid toxicity are increased by supplemental oxygen exposure (36). The

lung was reported to tolerate hypoxia better than hyperoxia (37). A protective effect of hypoxia was implicated in reducing hyaline membrane changes of the lungs, but there was no change in the mortality rate (38).

N-Aceytl-L-cysteine (NAC)

Evidence of lipid peroxidation by paraquat in pulmonary tissue has been demonstrated (27). In paraquat poisoning, the redox reactions which occur lead to the formation of lipid hydroperoxides by peroxidation of polyunsaturated membranes by dismutating singlet oxygen. Antioxidants, such as vitamin E, are presumed to terminate the chain reaction of lipid peroxidation (24). The lipid hydroperoxides are finally reduced by gluthathione peroxidase to less toxic lipid alcohols by oxidizing reduced glutathione (GSH) and hence preventing lipid free radicals that lead to membrane damage (Fig 2).

There is a decrease in GSH in the liver in acute paraquat poisoning (28) indicating an important role in reducing hydroperoxide formation (Fig 1); therefore, significant depletion of GSH enhances paraquat toxicity. On the other hand, replenishing glutathione exogenously has not proved effective in diminishing the toxicity because GSH does not penetrate cell membranes readily (27,28). It is, however, proposed that NAC can serve as a source for replenishing GSH from its oxidized form and thus accelerates the conversion of the toxic lipid hydroperoxides to less toxic lipid alcohols.

Niacin

Niacin is beneficial to rats poisoned by paraquat (39). This finding was developed from earlier works which disclosed that the common sites of damage are at the enzyme level in bacteria poisoned by paraquat and by hyperbaric oxygen. The evidence included the discovery that niacin and thiamine were beneficial for the growth of E. coli poisoned by hyperbaric oxygen or by paraquat. The mechanism of thiamine protection remains unknown, but there is evidence that niacin protects E. coli because it circumvents the consequences of the poisoning of quinolinate phosphoribosyl transferase. This enzyme is required for the de novo synthesis of nicotinamide adenine dinculeotide (NAD). This led to the belief that the results may apply to higher life forms.

From experimental results (39), niacin-treated rats showed delayed and reduced dyspnea, and the death rate was reduced from 75% to 55%. The benefit of niacin is consistent with the demonstrated role of niacin in preventing cellular decreases of NAD during poisoning of bacteria by paraquat and by hyperbaric oxygen.

NATURE OF COLLAGEN

Collagen is a fibrous protein which is composed of individual polypeptides which are often laterally crosslinked by several types of bonding. Because of their structure, they are physically tough and water insoluble, valuable properties for their role as structural elements of tissue. Collagen, unlike other fibrous proteins, has no α-helix because of its high content of glycine (25%) and proline and

hydroxyproline (25% combined). However, since these particular amino acids occur with repeated regularity in the sequence, the polypeptide chains are wrapped around each other in cable fashion and crosslinked by hydrogen bonding to form tropocollagen, the basic structural unit of collagen.

THE EFFECTS OF INHALATION OF PARAQUAT

Exposure of rats to aerosols of paraquat containing over 80% droplets in the size range of 2.5—5 μm produced pathological changes in lungs (6). Pulmonary epithelium was damaged, and there were pulmonary capillary congestion, extensive alveolar edema, fibrin and hemorrhages following aerosolizations. The rats usually made a complete recovery if death did not supervene three days after the exposure. It was impossible to produce pulmonary fibrosis by respirable aerosols of paraquat despite the extensive pulmonary damage (6).

In a further investigation (40), paraquat was tracheally instilled into rats using Indian ink marker. Only localized fibrosis and epithelial proliferation was described; the rest of the lungs remained normal.

METHODS OF PARAQUAT AND DIQUAT ASSAY

The concentration of the bipyridyls in the blood and urine of paraquat-exposed patients is used to estimate the dose and the rate of clearance from the body. A method of assaying paraquat and diquat utilizes their colored stable free-radicals (16). In this technique, measured quantity of urine from a paraquat patient is diluted to 50 ml with water and the protein is precipitated by adding

trichloroacetic acid. This mixture is centrifugated and the pellet is re-extracted with trichloroacetic acid. The supernatant of both solutions is pooled and passed through a cation exchange resin. For paraquat the resin is left in its hydrogen form, while the column is converted to a sodium form for diquat by washing with sodium chloride. The bipyridyls are eluted from the column by a mixture of 5 ml ammonium chloride and 1 ml freshly prepared sodium dithionite. Then 1 N sodium hydroxide is added to the eluate. This reduces the bipyridyl ion to its colored radical. Using a light wave length of 600 nm, the optical absorbance of the colored solution can be measured spectrophometrically. This is then compared with calibration graphs prepared from standard solutions. As little as 3 ug diquat and 10 ug paraquat may be detected by this method.

A similar and faster method of assay can be carried out in which 2 ml of 1% sodium dithionite in 1 N sodium hydroxide is added to 10 ml of urine. A blue coloration detectable with the naked eye is indicative of the presence of the bipyridyls.

These two techniques have been applied to measure the concentration of paraquat in the blood and urine of human cases of paraquat poisoning and hence to calculate the amount of paraquat excreted daily.

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Fig 1. Proposed mechanism of action for the in vivo toxicity of paraquat in which paraquat is reduced by microsomal NADPH-cytochrome reductase (27).

Fig 2. Proposed role of N-acetyl-L-cysteine (NAC) in the reduction of paraquat poisoning. Adapted from Bus et al (27). PUFA = Polyunsaturated Fatty Acid.

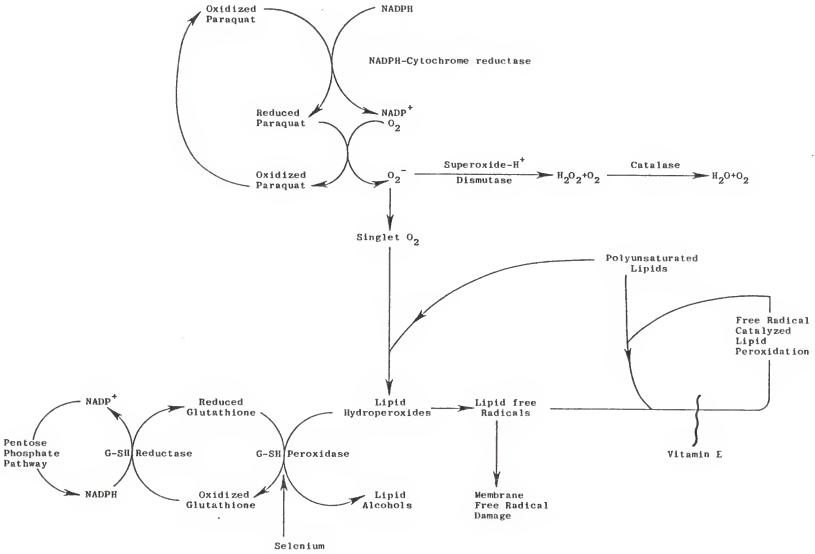
Oxidized Paraquat
NADPH
NADPH-Cytochrome reductase
Reduced Paraquat
NADP+
O2
Oxidized Paraquat
O2-
Superoxide-H+ Dismutase
H2O2+O2
Catalase
H2O+O2
Singlet O2
Polyunsaturated Lipids
Free Radical Catalyzed Lipid Peroxidation
NADP+
Reduced Glutathione
Lipid Hydroperoxides
Lipid free Radicals
Pentose Phosphate Pathway
G-SH Reductase
G-SH Peroxidase
Vitamin E
NADPH
Oxidized Glutathione
Lipid Alcohols
Membrane Free Radical Damage
Selenium

Fig 1.

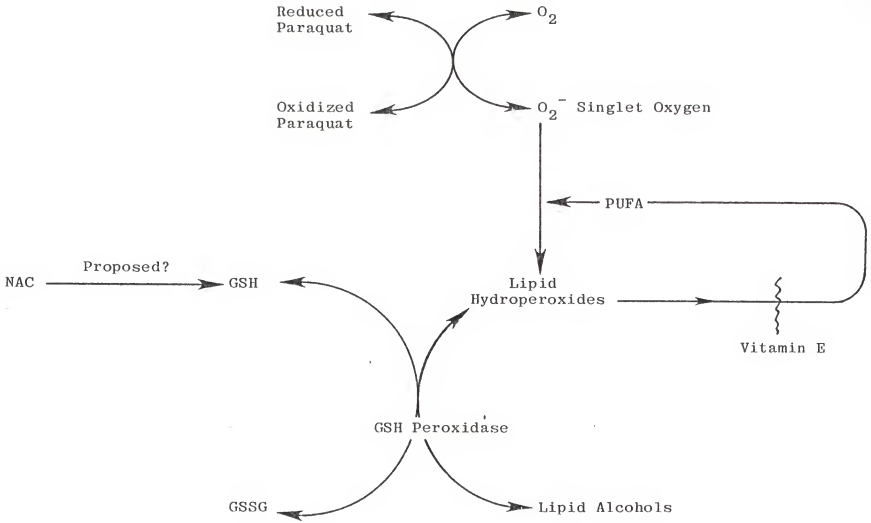
Reduced
Paraquat
O_2
Oxidized
Paraquat
O_2^- Singlet Oxygen
PUFA
Proposed?
NAC
GSH
Lipid
Hydroperoxides
Vitamin E
GSH Peroxidase
GSSG
Lipid Alcohols

Fig 2

APPENDIX II

TREATMENT OF RATS WITH PARAQUAT AND/OR THE COLLAGEN INHIBITORS L-3,4-DEHYDROPROLINE (L-DHP) AND DL-3,4-DEHYDROPROLINE (DL-DHP)

(A review and summary of the previous studies of F. Akahori and F.W. Oehme: Inhibition of Collagen Synthesis as a Treatment for Paraquat Poisoning. Vet Hum Toxicol 25:321-327, 1983.)

In a preliminary study male Sprague-Dawley rats were used. Nine groups of six rats each (54 total) were exposed to varying doses of paraquat (1 mg/kg to 50 mg/kg body weight) subcutaneously to determine the dose effective in producing pulmonary fibrosis. This study established that 7 mg/kg of paraquat dichloride solution given subcutaneoulsy for 7 or 8 days was needed to initiate pulmonary fibrosis. The loss in body weight of the rats and the increase in lung hydroxproline content were the criteria for this determination; when a decrease in body weight was observed paraquat administration was discontinued.

Of a total of 134 previously untreated rats, 31 received 1.4 ml/kg body weight of sterile saline solution and 103 rats received 7 mg paraquat/kg body weight for 7 or 8 days (Fig. 1). All the doses were given subcutaneously. Ten days after the start of paraquat dosing (day 10), paraquat-dosed group of rats was divided randomly into three groups to receive L-3,4-dehydroproline (L-DHP) or DL-3,4-dehydroproline (DL-DHP) or saline; 34 rats received 25 mg L-DHP/kg/day, 34 rats received 25 mg DL-DHP/kg/day, and 35 rats received 1.4 ml sterile saline/kg/day, each for 14 consecutive days (days 11-24). The saline-dosed group of rats also received saline injection subcutaneously from days 11-24 of the 103 rats that received paraquat, 31 died within days 11-24 — 13 in the paraquat/L-DHP group, 9 in the paraquat/DL-DHP and 9 in the paraquat/saline group.

Rats from the various groups (saline/saline, paraquat/L-DHP, paraquat/DL-DHP, paraquat/saline) were sacrificed on days 10, 18, 25, 32, 39 and 100 after the paraquat dosing was stopped for the determination of lung and heart weights, total lung protein and collagen

content of the lung and to collect lung tissues for histopathology. Sacrifice was induced using methoxyflurane and the rats were drained of blood via the abdominal aorta. The lung, blood and heart samples were preserved by rapid freezing in liquid nitrogen for biochemical analysis while the right posterior caudal lobe of the lung was fixed in formalin for later morphometric histopathology.

An increase in the body weights of paraquat-treated rats from day 25 to day 39 was observed. Paraquat/L-DHP and paraquat/DL-DHP-treated groups gained slightly more weight than the paraquat/saline-treated group between days 32 and 39. This difference was not statistically significant. The mean lung wet weight of the rats that gained weight was not statistically significant between groups. However, the lung wet weight to body weight ratios in the paraquat-treated animals whose weight decreased was statistically significant at the 1% and 5% level on days 10 to 39. No significant differences were observed in the heart weights of all the groups of rats. The lung total protein concentrations in the paraquat-treated groups increased significantly (at the 5% level). The total lung collagen of the paraquat-treated rats, expressed as umol of hydroxyproline per rat, was significantly increased (at the 1% level) on day 10. Collagen accumulation in the lungs of the paraquat/saline-treated rats was significantly higher (at the 1% and 5% level) during the 39 days of observation. L-DHP or DL-DHP administration did not significantly block the rise in lung collagen content as shown by comparing paraquat-treated rats which did not receive L-DHP or DL-DHP (paraquat/saline) to those that did. All paraquat-dosed rats had markedly increased lung collagen levels compared

to the saline-dosed animals (saline/saline group). The lung collagen content of rats treated with DL-DHP (paraquat/DL-DHP) was less elevated than in the rats that did not receive DL-DHP (paraquat/saline). This difference, however, was not statistically significant. No paraquat-induced pulmonary fibrosis was fatal during day 39-100.

The effect of L-DHP or DL-DHP on inhibition of lung collagen synthesis was not significant. These "antidotes" failed to alter pulmonary fibrosis resulting from paraquat expsoure and intoxication to rats.

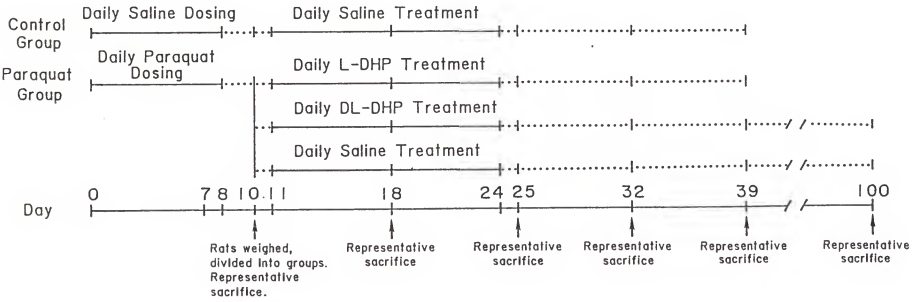

Fig 1. Experimental design for the study of lung collagen synthesis inhibition by dehydroproline (L-DHP or DL-DHP) following paraquat exposure in rats. Groups are designated saline/saline, paraquat/L-DHP, paraquat/DL-DHP and paraquat/saline.

APPENDIX III

INDIVIDUAL RESULTS OF RAT LUNG MORPHOMETRIC MEASUREMENTS

FIGURE CAPTIONS

Fig 1 -- Extensive collapse of lung alveoli and marked consolidation of parenchyma. Paraquat/Saline Group. Day 10. Mag x 330.

Fig 2 -- Average number of intra-alveolar macrophages on various days after initiation of paraquat dosing.

Fig 3 -- Average number of mononuclear cells on various days after initiation of paraquat dosing.

Fig 4 -- Average number of total pulmonary cells on various days after paraquat dosing was initiated.

Fig 5 -- Mean alveolar wall thickness (μm) on various days after paraquat dosing was initiated.

Fig 6 -- Average lung consolidation (%) on various days after paraquat dosing was initiated.

Fig 7 -- Type I pneumocytes (%) on various days after paraquat dosing was started.

Fig 8 -- Type II pneumocytes (%) on various days after paraquat dosing was started.

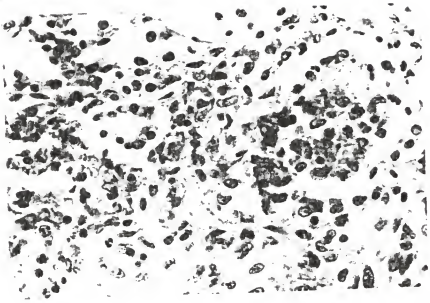

Fig 1.

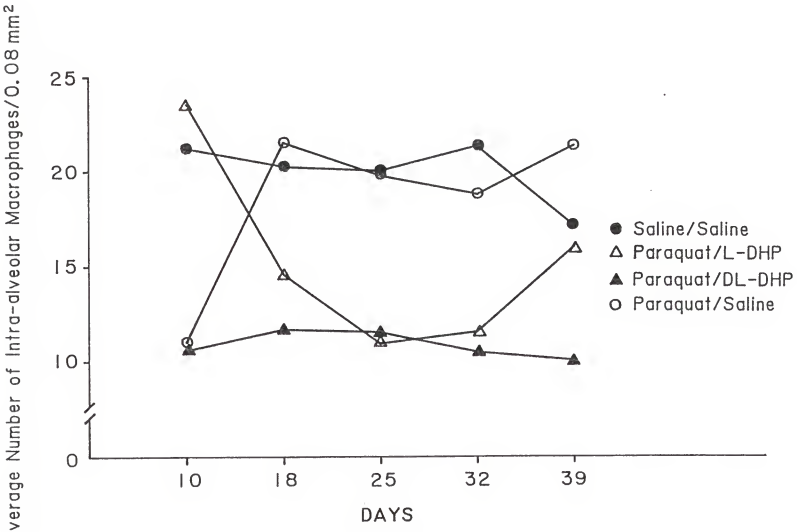
Average Number of Intra-alveolar Macrophages/0.08 mm²
25
20
15
10
0
10
18
25
32
39
DAYS
Saline/Saline
Paraquat/L-DHP
Paraquat/DL-DHP
Paraquat/Saline

Fig 2.

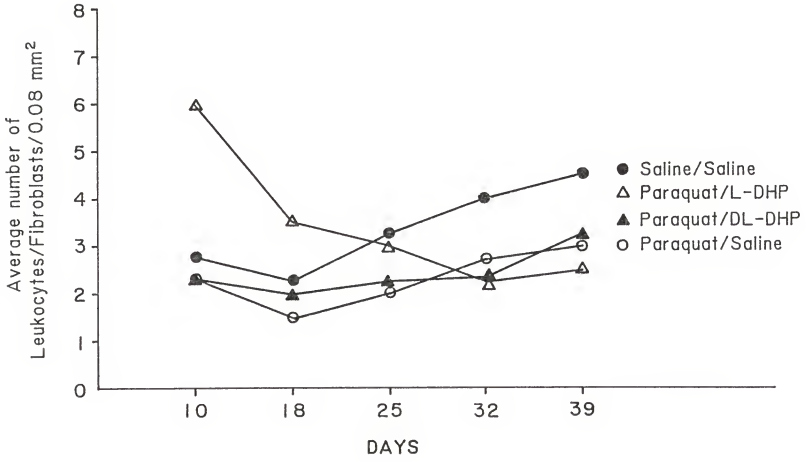
Average number of
Leukocytes/Fibroblasts/0.08 mm²
8
7
6
5
4
3
2
1
0
10
18
25
32
39
DAYS
Saline/Saline
Paraquat/L-DHP
Paraquat/DL-DHP
Paraquat/Saline

Fig 3.

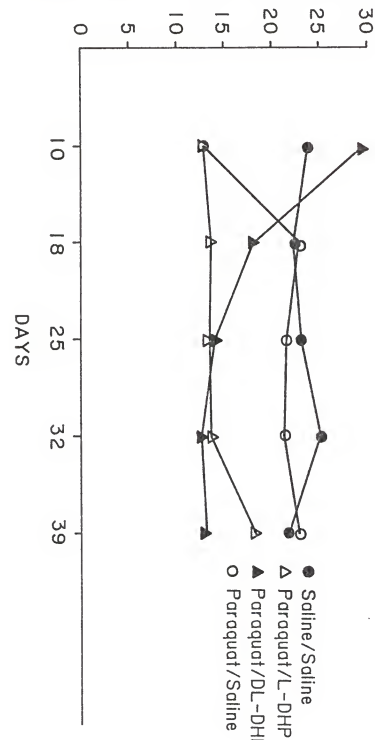
Average Number of Total Pulmonary Cells/0.08 mm²
30
25
20
15
10
5
0
10
18
25
32
39
DAYS
● Saline/Saline
△ Paraquat/L-DHP
▲ Paraquat/DL-DHP
○ Paraquat/Saline

Fig 4.

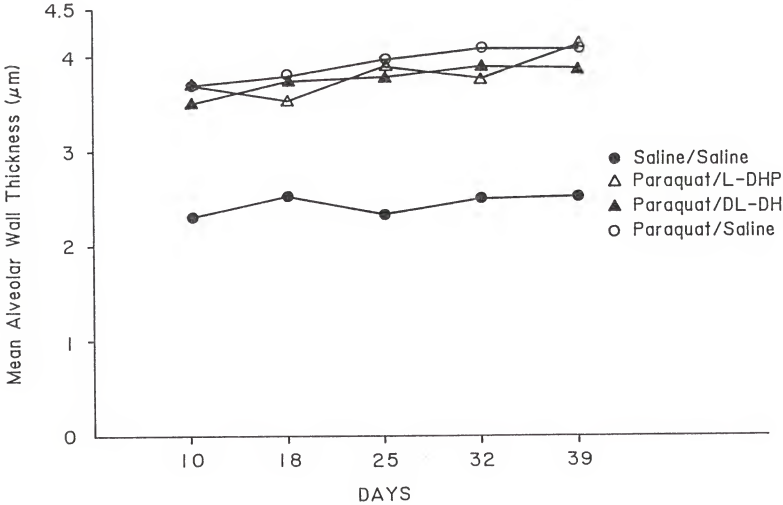
4.5
4
3
2
1
0
Mean Alveolar Wall Thickness (μm)
10
18
25
32
39
DAYS
Saline/Saline
Paraquat/L-DHP
Paraquat/DL-DHP
Paraquat/Saline

Fig 5.

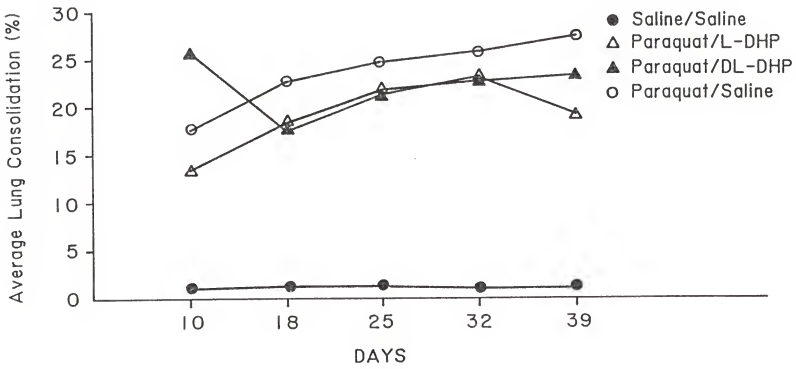
Average Lung Consolidation (%)
30
25
20
15
10
5
0
10
18
25
32
39
DAYS
Saline/Saline
Paraquat/L-DHP
Paraquat/DL-DHP
Paraquat/Saline

Fig 6.

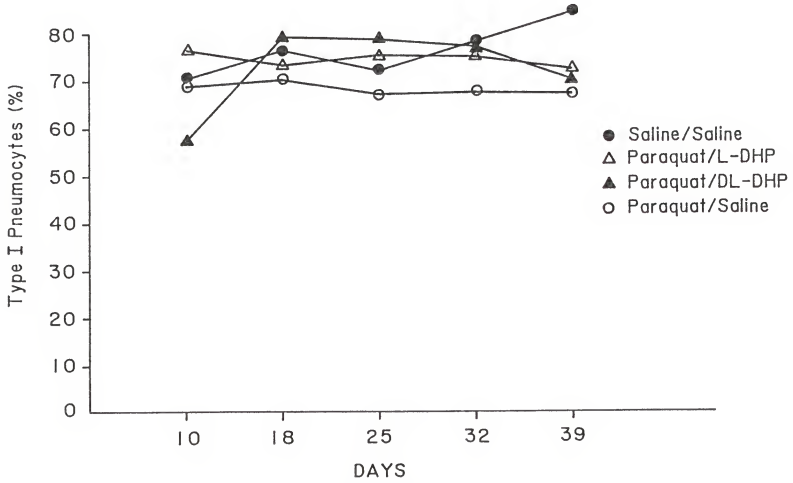
Type I Pneumocytes (%)
80
70
60
50
40
30
20
10
0
10
18
25
32
39
DAYS
Saline/Saline
Paraquat/L-DHP
Paraquat/DL-DHP
Paraquat/Saline

Fig 7.

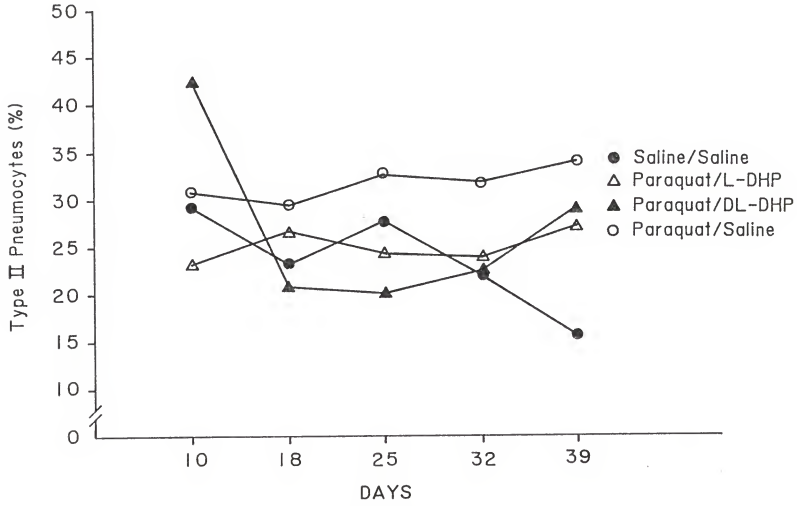
50
45
40
35
30
25
20
15
10
0
Type II Pneumocytes (%)
10
18
25
32
39
DAYS
Saline/Saline
Paraquat/L-DHP
Paraquat/DL-DHP
Paraquat/Saline

Fig 8.

Table 1: Individual rat lung morphometric measurement of various parameters from rats that received saline only during both the dosing and the treatment period (control group: saline/saline). Each person represents the mean of thirty separate microscopic cell counts or tissue measurements/0.08 mm^2 field of view.

Rat Number	Day of Sacrifice After Dosing Was Started	No. of Intra-alveolar Macrophages	No. of Leucocytes/ Fibroblasts	Total No. of Pulmonary Cells	Alveolar Wall Thickness (μm)	Consolidation of Lung Parenchyma (%)	Type I Pneumocytes (% of Total Pneumocytes)	Type II Pneumocytes (% of Total Pneumocytes)
112	10	24	2	26	2.75	1	66.7	33.3
129	10	20	3	23	2.50	1	66.7	33.3
145	10	23	3	26	2.00	1	66.7	33.3
150	10	18	3	21	1.96	1	83.3	16.7
104	18	20	2	22	2.60	1	85.7	14.3
137	18	21	2	23	2.50	2	71.4	28.6
140	18	22	3	25	2.65	1	66.7	33.3
174	18	18	2	20	2.42	1	83.3	16.7
123	25	21	3	24	2.65	1		
135	25	20	3	23	2.65	1	83.3	16.7
148	25	19	3	22	1.98	1	66.7	33.3
168	25	20	4	24	2.25	2	66.7	33.3
117	32	23	2	25	2.65	2	66.7	33.3
157	32	24	4	28	2.25	1	83.3	16.7
164	32	19	3	22	2.50	1	85.7	14.3
183	32	19	7	26	2.65	1	66.7	33.3
103	39	19	3	22	2.80	1	85.7	14.3
142	39	19	1	10	2.42	1	83.3	16.7
161	39	19	7	26	2.42	1	85.7	14.3
162	39	14	7	21	2.50	1	83.3	16.7

Table 2: Individual rat lung morphometric measurement of various parameters from rats that received 7 mg paraquat/kg body weight during the 7-8 days dosing period and 25 mg L-3,4-dehydroproline (L-DHP)/kg/day during the treatment period of 14 days (day 11-24) (paraquat group: paraquat/L-DHP). Each value represents the mean of thirty separate microscopic cell counts or tissue measurements/0.08 mm^2 field of view.

Rat Number	Day of Sacrifice After Dosing Was Started	No. of Intra-alveolar Macrophages	No. of Leucocytes/ Fibroblasts	Total No. of Pulmonary Cells	Alveolar Wall Thickness (μm)	Consolidation of Lung Parenchyma (%)	Type I Pneumocytes (% of Total Pneumocytes	Type II Pneumocytes (% of Total Pneumocytes
122	10	22	8	30	ND	ND	ND	ND
133	10	29	5	34	4.41	5	83.3	16.7
153	10	26	10	36	3.43	8	ND	ND
165	10	27	5	32	3.43	10	80.0	20.0
178	10	14	2	16	3.43	80	66.7	33.3
110	18	18	5	23	3.43	27	66.7	33.3
120	18	13	1	15	3.43	14	83.3	16.7
156	18	13	3	16	3.43	25	60.0	40.0
159	18	14	4	18	3.92	14	83.3	16.7
111	25	12	1	13	3.92	21	83.3	16.7
151	25	9	3	12	3.92	17	66.7	33.3
171	25	11	2	13	3.43	23	83.3	16.7
180	25	12	6	18	4.41	25	70.0	30.0
119	32	10	3	13	3.79	22	77.8	22.2
160	32	12	2	13	3.92	20	60.0	40.0
169	32	13	2	15	3.43	24	83.3	16.7
172	32	11	2	13	3.92	26	83.3	16.7
124	39	15	2	17	3.92	5	85.7	14.3
126	39	25	2	27	4.41	22	66.7	33.3
176	39	10	2	12	3.92	25	66.7	33.3
179	39	14	4	18	4.41	25	71.4	28.6

Table 3: Individual rat lung morphometric measurement of various parameters from rats that received 7 mg paraquat/kg body weight during the 7-8 days dosing period and 25 mg DL-3,4-dehydroproline (DL-DHP)/kg/day during the treatment period of 14 days (days 11-24) (paraquat group: paraquat/DL-DHP). Each value represents the mean of thirty separate microscopic counts or tissue measurements/0.08 mm^2 field of view.

Rat Number	Day of Sacrifice After Dosing Was Started	No. of Intra-alveolar Macrophages	No. of Leucocytes/ Fibroblasts	Total No. of Pulmonary Cells	Alveolar Wall Thickness (μm)	Consolidation of Lung Parenchyma (%)	Type I Pneumocytes (% of Total Pneumocytes)	Type II Pneumocytes (% of Total Pneumocytes)
114	10	11	3	14	3.43	22	ND	ND
132	10	12	3	15	3.75	15	ND	ND
175	10	ND	ND	ND	3.92	13	ND	ND
182	10	9	1	10	2.94	4	57.1	42.9
128	18	12	4	16	4.41	25	83.3	16.7
138	18	10	2	12	3.75	15	83.3	16.7
152	18	13	1	14	3.43	25	66.7	33.3
177	18	12	1	13	3.43	9	83.3	16.7
102	25	11	2	13	4.41	16	83.3	16.7
109	25	14	2	16	3.92	18	77.8	22.2
130	25	12	2	14	3.43	25	83.3	16.7
143	25	9	3	12	3.43	28	71.4	28.6
113	32	12	3	15	3.92	25	66.7	33.3
134	32	0	2	11	3/43	28	83.3	16.7
146	32	10	2	12	4.41	15	83.3	16.7
121	39	7	4	11	3.79	27	66.7	33.3
141	39	12	2	14	3.92	18	66.7	33.3
154	39	11	4	15	3.92	23	83.3	16.7
167	39	10	3	13	3.92	25	66.7	33.3
147	39	11	2	13	3.92	25	66.7	33.3
185	39	15	2	17	4.65	27	60.0	40.0
125	39	22	2	24	3.92	25	62.0	38.0
28	100	8	1	9	4.65	33	50.0	50.0
29	100	10	2	12	4.41	32	60.0	60.0
31	100	12	2	14	4.90	30	71.4	28.6
34	100	10	3	13	3.92	35	66.7	33.3

Table 4: Individual rat lung morphometric measurement of various parameters from rats that received 7 mg paraquat/kg body weight during the 7-8 days dosing period and only saline during the 14 days treatment period (day 11-24) (paraquat group: paraquat/saline). Each value represents the mean of thirty separate microscopic cell counts or tissue measurements/0.08 mm^2 field of view.

Rat Number	Day of Sacrifice After Dosing Was Started	No. of Intra-alveolar Macrophages	No. of Leucocytes/ Fibroblasts	Total No. of Pulmonary Cells	Alveolar Wall Thickness (μm)	Consolidation of Lung Parenchyma (%)	Type I Pneumocytes (% of Total Pneumocytes)	Type II Pneumocytes (% of Total Pneumocytes)
108	10	10	2	12	3.43	18	66.7	33.3
115	10	12	3	15	3.92	7	71.4	28.6
186	10	11	2	14	ND	28	ND	ND
127	18	23	1	24	3.92	25	66.7	33.3
165	18	20	1	21	3.92	26	66.7	33.3
166	18	19	2	21	3.92	22	71.4	28.6
187	18	24	2	26	3.43	18	77.8	22.2
105	25	19	1	20	3.92	18	83.3	16.7
116	25	20	2	22	3.92	21	66.7	33.3
173	25	21	3	24	3.58	30	60.0	40.0
184	25	19	2	21	4.41	30	60.0	40.0
101	32	19	2	21	4.41	27	71.4	26.6
106	32	18	3	21	4.41	24	66.7	33.3
131	32	18	4	22	3.92	22	66.7	33.3
158	32	20	2	22	3.58	30	66.7	33.3
107	39	19	2	21	4.41	30	71.4	28.6
118	39	23	2	25	3.92	22	66.7	33.3
139	39	21	2	23	4.65	28	66.7	33.3
144	39	19	2	21	4.92	26	66.7	33.3
163	39	16	4	20	4.41	25	50.0	50.0
170	39	13	5	18	4.41	22	66.7	33.3
30	100	19	2	21	4.41	34	71.4	28.6
32	100	14	3	17	5.00	36	66.7	33.3
33	100	22	3	25	4.94	32	66.7	33.3
35	100	14	3	17	4.94	38	40.0	60.0

Table 5: The mean ± standard error values of the various parameters under consideration for the groups of experimental rats taken on days 10,18,25,32,39, and 100 after dosing 7-8 days with saline and then treated for 14 days (day 11-24) with saline (saline/saline group), or after dosing 7-8 days with paraquat (PQT) and then treated for 14 days (day 11-24) with L-DHP (PQT/L-DHP group), DL-DHP (PQT/DL-DHP group) or saline (PQT/saline group).

Group	Day of Sacrifice After Dosing Was Started	No. of Animals	*No. of Macrophages	*No. of Leucocytes/ Fibroblasts	*Total No. of Pulmonary Cells	*Alveolar Wall Thickness (um)	Consolidation of Lung Parenchyma (%)	*Relative No. of Type I Pneumocytes (%)	*Relative No. of Type II Pneumocytes (%)
Saline/Saline	10	4	21.2±1.37	2.8±0.74	24.0±1.61	2.3±0.17	1.0±4.82	70.8±4.08	29.2±4.12
	18	4	20.2±1.37	2.2±0.74	22.5±1.61	2.5±0.17	1.2±4.82+	76.8±4.08	23.2±4.12
	25	4	20.0±1.37	3.2±0.74	23.2±1.61	2.4±0.17	1.2±4.82+	72.2±4.71	27.8±4.75
	32	4	21.2±1.37	4.0±0.74	25.2±1.61	2.5±0.17	1.0±4.82+	78.4±4.08	21.6±4.12
	39	4	17.8±1.37	4.5±0.74	22.0±1.61	2.5±0.17	1.0±4.82+	84.5±4.08	15.5±4.12
PQT/L-DHP	10	4	23.6±1.23	6.0±0.66	29.6±1.44	3.7±0.17	25.8±4.82	76.7±4.71	23.3±4.75
	18	4	14.5±1.37+	3.5±0.74+	18.0±1.61+	3.6±0.17	17.5±4.82	73.3±4.08	26.7±4.12+
	25	4	11.0±1.37+	3.0±0.74+	14.0±1.61+	3.9±0.17	21.5±4.82	75.6±4.08	24.2±4.12
	32	4	11.5±1.37+	2.2±0.74+	13.8±1.61+	3.8±0.17	23.0±4.82	76.1±4.08	23.9±4.12
	39	4	15.0±1.37+	2.5±0.74+	18.5±1.61+	4.2±0.17	19.2±4.82	72.6±4.08	27.4±4.12+
PQT/DL-DHP	10	4	10.7±1.59	2.3±0.85	13.0±1.86	3.5±0.17	13.5±4.82	57.1±8.15	42.9±8.23
	18	4	11.8±1.37	2.0±0.74	13.8±1.61	3.8±0.17	18.5±4.82	79.2±4.08	20.8±4.12
	25	4	11.5±1.37	2.2±0.74	13.8±1.61	3.8±0.17	21.8±4.82	79.0±4.08	21.0±4.12
	32	3	10.3±1.59	2.3±0.85	12.8±1.86	3.9±0.20	22.7±5/57	77.3±4.71	22.2±4.75
	39	4	10.0±1.37	3.2±0.74	13.2±1.61	3.8±0.17	23.2±4.82	70.8±4.08	29.2±4.12
	39	3	13.0±1.37	2.0±0.74	15.0±1.61	4.3±0.17	26.0±4.82	63.4±7.32	36.6±5.57
	100	4	10.0±1.37	2.0±0.74	12.0±1.33	4.5±0.21	32.5±4.40	63.2±7.32	38.8±7.15
PQT/Saline	10	3	11.0±1.59	2.3±0.85	13.3±1.86	3.7±0.25	17.7±5.57	69.0±5.77	31.0±5.82
	18	4	21.5±1.37	1.5±0.74	23.0±1.61+	3.8±0.17	22.8±4.82	70.6±4.08	29.4±4.12
	25	4	19.8±1.37	2.0±0.74	21.8±1.61+	4.0±0.17	24.8±4.82	67.5±4.08	32.5±4.12
	32	4	18.8±1.37	2.8±0.74	21.5±1.61+	4.1±0.17	25.8±4.82	67.9±4.08	31.6±4.12
	39	4	21.2±1.37	2.5±0.74	23.0±1.61+	4.1±0.17	27.5±4.82	67.7±4.08	33.8±4.12
	39	3	17.5±1.37	3.2±0.74	20.7±1.61	4.2±0.17	25.0±4.82	65.5±3.58	35.5±4.64
	100	4	17.2±1.37	2.8±0.74	20.0±1.91	4.8±0.17	35.0±4.04	61.2±3.58	38.8±6.67

*Area of field of view for these parameters is 0.08 mm^2, excepting the consolidation of lung parenchyma and relative number of cell types (type I and type II pneumocytes), which are expressed in percent of total, all the other parameters are in micrometer (um).
+Value is statistically different at p 0.05 when compared to the day 10 value within the same group.

PULMONARY MORPHOMETRIC HISTOPATHOLOGY OF SALINE OR PARAQUAT-EXPOSED RATS TREATED WITH THE COLLAGEN INHIBITOR DEHYDROPROLINE

by

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AN ABSTRACT OF A MASTER'S THESIS

submitted in partial fulfillment of the
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Paraquat is a non-selective herbicide, expsoure to which has been associated with animal and human deaths. The most devastating effect of paraquat poisoning is the exudation of edema fluid and hemorrhages into the lung parenchyma. If the victim survives these initial effects, a fulminating fibrotic process develops and death results from anoxia due to progressive pulmonary fibrosis. Paraquat poisoned rats have increased lung levels of hydroxyproline and prolyl-hydroxylase, and an increased rats of collagen synthesis leading to pulmonary fibrosis. The proline analogue dehydroproline (DHP) inhibits the biosynthesis and secretion of collagen by competitively blocking the aminoacylation of t-RNA by proline. DHP also inhibits prolyl-hydroxylase activity. Because of these effects on collagen biosynthesis, DHP was studied as an antidote for experimental paraquat poisoning in rats.

Preliminary studies determined that the subcutaneous administration of 7 mg paraquat/kg body weight for 7-8 days was an appropriate dosage for this study. A decrease in the body weight of the rats between days 1 and 10 after paraquat dosing was started, was used to detect rats in which paraquat had induced pulmonary fibrosis. L-DHP or DL-DHP at a dose rate of 25 mg/kg body weight or saline at a dose rate of 1.4 ml/kg body weight was then administered subcutaneously to groups of the paraquat-dosed rats for 14 days (day 11-24). One group of rats that earlier received no paraquat, but only saline, was treated with saline for 14 days as a negative control group. Representative numbers of rats from each group were sacrificed on days 10, 18, 25, 32, 39 and 100. Lung tissue was collected and fixed in formalin for histopathological preparation. Morphometric histopathological measurements and

analysis were applied to the following parameters under light microscopy: number of intra-alveolar macrophages and leucocytes, total number of pulmonary cells, mean alveolar wall thickness, percent consolidation of lung parenchyma and the relative number of various cell types (type I and type II pneumocytes). The results were compared between the various groups using two-way analysis of variance.

Although statistical analysis of the various parameters showed no improvement of pulmonary fibrosis or consolidation with DHP treatment after paraquat intoxication, DHP did significantly reduce the total number of pulmonary cells, especially the number of macrophages. If stimulation of fibroblastic activity is through the medium of macrophage number, DHP may have in this sense decreased the lung response to paraquat exposure. However, the administered DHP did not reduce the accompanying pulmonary consolidation and fibrosis.