EFFECT OF PARAQUAT ON PLASMA FIBRONECTIN, SERUM FREE HYDROXYPROLINE, SERUM CERULOPLASMIN AND LUNG COLLAGEN CONTENT IN MONKEYS

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Accepted April 7, 1987

Abstract.....The purpose of this study was to characterize paraquat toxicity in monkeys and to determine the feasibility of using the monkey as an animal model for the study of paraquat-induced pulmonary fibrosis in humans.

Sixteen Japanese monkeys (*Macaca fuscata*), more than 3.5 years of age, with bodyweight ranging from 3.2 kg to 10.2kg, were randomly divided into two groups. They were administered paraquat dichloride (PQ) by injection (s.c.) at a dose of 2.0 mg/kg bodyweight (12 monkeys) or s.c. saline, at a dose of 0.2 ml/kg bodyweight (control group, 4 monkeys), every two days for a period of four to five times.

Eight monkeys (66.7%) from the PQ treatment groups expired due to subchronic PQ toxicity from days 11 to 35. Four monkeys (33.3%) from the PQ treatment group and all four monkeys from the control group survived the observation period of 66 days. On day 66, all of the surviving monkeys were sacrificed and examined for possible histopathological changes and the lung hydroxyproline content was determined.

Our results indicate that the concentration of free hydroxyproline and plasma fibronectin did not vary significantly. The serum ceruloplasmin for the monkeys of the PQ treatment groups was significantly increased from day 14 to 21, compared to the control group. Also the total lung collagen of both the expired and surviving monkeys in the PQ treatment groups was elevated significantly, compared to the control group. The monkeys can provide extensive opportunities for research on the mechanism and the treatment of PQ poisoning in man.

Key words : Ceruloplasmin, Fibronectin, Free hydroxyproline, Hydroxyproline, Lung collagen, Lung hydroxyproline, Monkey, Paraquat, Pulmonary fibrosis, Serum hydroxyproline.

INTRODUCTION

Paraquat (PQ) is an effective herbicide and defoliant used throughout the world. Increased exposure to PQ has contributed to a growing problem of PQ related illness and death in humans (Bullivant, 1966 ; Fitzgerald et al., 1970 ; Naito et al., 1984). Death, occurring during the initial phase of acute PQ poisoning in man is usually due to shock resulting from mucosal erosion, esophageal perforation, pulmonary edema, pulmonary hemorrhage and adrenal disorders (Fletcher, 1972 ; Adachi et al., 1978 ; Miyamoto et al., 1983). If the victim survives this initial acute phase, a fibrotic process usually develops, the speed and severity of which depends upon the exposure dose and death follows, which is usually attributable to respiratory insufficiency (Fletcher, 1972 ; Heath et al., 1977 ; Yamauchi et al., 1982).

This process is very similar for most animals and man. Although pulmonary fibrosis in rats (Akahori et al., 1983) was induced by PQ, it was not fatal during the PQ study observation periods of 100 days. Using dogs as his experimental animal model, Akahori et al. (1985) found that pulmonary fibrosis was difficult to induce using PQ. Moreover, PQ-induced pulmonary fibrosis has not been observed in rabbits (Butler et al., 1971 ; Ilett et al., 1974) or guinea pigs (Murray et al., 1972).

Thus there are both significant species and individual variations in the degree of pulmonary fibrosis elicited by PQ among rats, dogs, other animals and man. The purpose of this study was to characterize PQ toxicity in monkeys and to evaluate the suitability of using the monkey as an animal model for the study of PQ-induced pulmonary fibrosis in humans.

MATERIALS AND METHODS

Animals and Drugs

Sixteen male and female Japanese monkeys (*Macaca fuscata*), more than 3.5 years of age, with bodyweights ranging from 3.2 kg to 10.2 kg, were observed for 6 to 12 months after their arrival from our supplier. These monkeys were placed in individual stainless steel cages, under controlled conditions of 22°C ($\pm 5^\circ\text{C}$) and relative humidity of 65% ($\pm 14\%$). They were fed a commercial solid diet (Oriental's Monkey Diet, from Oriental Yeast Ind., Chiba, Japan), with water, ad libitum. The lighting schedule was 12 hours light and 12 hours dark (12L : 12D), with the lights turned on at 0600. The paraquat used in our experiment was 1, 1'-dimethyl-4, 4'-bipyridilium dichloride. The paraquat dichloride (purity 98.3%) was a gift from Dr. Teruyuki Misumi, Asahi Chemical Industry, Kawasaki, Japan.

Dosing of the Monkeys

In a preliminary study, a dose of 2.0 mg/kg bodyweight, every two days for 4–5 times produced some acute or subchronic toxicity during and after the period of PQ dosing. This dosing schedule was used for this experiment.

The animals were randomly divided into two PQ dosage groups with different dosing schedules and a control group (Fig. 1). Paraquat dichloride (PQ), dissolved in physiological saline was subcutaneously injected four times (Group I, 8 monkeys) and five times (Group II, 4 monkeys) using a standard PQ dose of 2 mg/kg bodyweight, every two days. The control group (Group III, 4 monkeys) received subcutaneous injections of 0.2 ml/kg bodyweight of saline, five times every two days. Blood samples were collected on days 1, 3, 5, 7, 9, 14, 21, 34 and 66 for the determination of plasma fibronectin, serum free hydroxyproline and serum ceruloplasmin levels. On day 66, the four surviving monkeys from the PQ-treatment groups and all four monkeys from the control group were sacrificed and the lung collagen content was determined.

Fig. 1 Dosing and sampling schedules

Biochemical Determinations

The animals were anesthetized with halothane and blood samples were collected using a plastic syringe with a venoneedle. The blood specimens consisting of a 2 ml and a 5 to 8 ml portions were transferred into test tubes containing 1.0 mg of disodium ethylene-diaminetetraacetate (EDTA- Na_2) and a plain test tube, without anticoagulants, respectively. The blood specimens were then centrifuged and the plasma and serum separated. The samples were rapidly frozen in Dewar flasks with dry ice and isopropanol and then stored at -80°C until they were assayed. The left caudal lobe of the lung was also rapidly frozen using dry ice and stored at -80°C until it was also assayed.

Plasma Fibronectin Concentration

The plasma fibronectin was assayed using the Fibronectin, Opsonic Protein, Turbidimetric Immuno-Assay UV-Test (Boehringer Mannheim Biochemica, W. Germany).

Serum Ceruloplasmin Level

The serum ceruloplasmin levels were measured using the method of Ravin et al. (1956), modified according to Rice (1962).

Serum Free Hydroxyproline Level

The serum free hydroxyproline levels were measured using the method initially described by Stegemann (1958) and later modified by Fujii et al. (1981).

Lung Collagen Content

The collagen content of the lung was measured from the frozen left caudal lung samples which were first thawed and weighed. These specimens were minced and then homogenized for 2 minutes at 4°C in sufficient deionized water to yield an approximate 10% mixture (wt/vol). The lung hydroxyproline content was determined from the amount of collagen in the aliquot from the left caudal lobe and assayed using the method of Jamall et al. (1981).

Statistics

The group data obtained was expressed as the mean and the standard error of the mean. All of the data was subjected to the Student's t-test or Welch's t-test for the determination of possible statistical significance. The significance level was established as $p \leq 0.05$.

RESULTS*Effect of PQ on Mortality*

Table 1 lists mortality. A total of 12 animals received the dose of 2.0 mg/kg of PQ, administered 4 or 5 times, every two days. In Group I, 5 monkeys (5/8, 62.5%) died from day 13 to 35. A mortality rate of 75% (3/4) was found in Group II, during the period from day 11 to 18. Therefore, 8 monkeys (8/12, 66.7%) from the PQ treatment groups expired due to subchronic or chronic PQ toxicity from day 11 to 35. The remaining 4 monkeys (4/12, 33.3%) from the PQ treatment groups survived the observation period of 66 days.

Table 1. Mortality of monkeys receiving paraquat (PQ)

Group	Number of monkeys	PQ-treated (s.c., every 2 days)	Mortality		Mortality rate(%)
			Days	Number of expired monkeys	
I	8	2 mg/kg, for 4 times	13—35	5	62.5
II	4	2 mg/kg, for 5 times	11—18	3	75.0
III	4	0.2 ml of saline, for 5 times	—	0	0

Effect of Paraquat on the Plasma Fibronectin Concentration

The plasma fibronectin concentrations for Group I (PQ-treated 4 times) increased on days 5 and 7 and both of the PQ treatment groups (Group I and Group II) demonstrated decreased fibronectin concentrations on days 1, 9 and 14, respectively. However, no significant differences between these groups was detected, possibly due to the large individual variations and the small sampling size of the animal groups in our study (Fig. 2).

Fig. 2 Daily variations in plasma fibronectin concentrations after PQ administration. Each point represents the mean $\pm$ S. E. M.

Group I (2 mg/kg PQ, for 4 times) —○—
 Group II (2 mg/kg PQ, for 5 times) —□—
 Group III (saline, control group) —●—

Effect of Paraquat on the Serum Free Hydroxyproline Level

The serum free hydroxyproline levels in the PQ treatment groups were lower than in the control group before PQ administration. However, no significant differences between these groups was detected ($p=0.05$). In Group I (2 mg/kg PQ given four times), the serum free hydroxyproline increased on day 14 compared to the control group and the other PQ treatment group. No significant differences between these groups was detected, again possibly due to the large individual variations observed in the free hydroxyproline levels (Fig. 3).

Effect of Paraquat on the Serum Ceruloplasmin Level

The serum ceruloplasmin levels are shown in Figure 4. In all of the PQ treatment groups, the serum ceruloplasmin was increased significantly compared to the control group on day 14 at the 5% level of significance and on day 21 at the 1% level of significance, respectively, after PQ administration. Indeed a significant four-fold increase was detected in the serum ceruloplasmin levels of the PQ treatment groups on the 21st day post PQ administration, with a subsequent return to the control (normal) levels.

Fig. 3 Daily variations in the serum free hydroxyproline levels after PQ administration. Each point represents the mean $\pm$ S. E. M.

Group I (2 mg/kg PQ, for 4 times) —○—
 Group II (2 mg/kg PQ, for 5 times) —□—
 Group III (saline, control group), —●—

Fig. 4 Daily variations in the serum ceruloplasmin levels after PQ administration. Each point represents the mean $\pm$ S. E. M. 1.0 mU represents the rate of oxidation of 1.0 nmol of the substrate, p-phenylenediamine/min/ml of serum.

Group I (2 mg/kg PQ, for 4 times) —○—
 Group II (2 mg/kg PQ, for 5 times) —□—
 Group III (saline, control group) —●—

Effect of Paraquat on the Lung Collagen Content

For all of our experimental groups, Group I (PQ treated, four times), Group II (PQ treated, five times) and the control group, the lung collagen content (expressed as mg of hydroxyproline/gram of lung tissue ($\bar{x} \pm \text{S.E.}$)) of each group was 1.54 ± 0.258 mg/g lung tissue, 1.52 ± 0.240 mg/g lung tissue and 0.68 ± 0.048 mg/g lung tissue, respectively. Thus, the PQ treatment groups had significantly higher (5% level of significance) lung collagen content than the saline treated animals (Fig. 5).

Fig. 5 Lung hydroxyproline content (Hyp.) after PQ administration. Lung hydroxyproline levels were measured for expired and/or sacrificed animals from day 11 to 35 and day 66. Each bar represents the mean $\pm$ S. E. M. Numbers in parentheses indicate the number of monkeys in each group. An asterisk indicates values that are significantly different from the control group (Group III).

Group I (2 mg/kg PQ, for 4 times)

Group II (2 mg/kg PQ, for 5 times)

Group III (saline, control group)

Figure 6 shows the relationship between the serum free hydroxyproline and the lung hydroxyproline concentrations. Monkeys that expired after PQ administration had markedly increased lung collagen levels when compared to the saline treated animals (control group) during the 66 day observation period. On the other hand, the serum free hydroxyproline concentrations of the PQ-treated monkey who died on day 15 (1 monkey) and one monkey that survived (during the 66 day observation period) were increased when compared to the control group and the other PQ-treated animals. Thus no correlation ($r=0.00136$) was detected between the free serum hydroxyproline and the lung hydroxyproline levels.

Fig. 6 Relationship between the free hydroxyproline concentration and the lung hydroxyproline content.

- Group III (control group, saline, sacrificed) ●
- Group I (2 mg/kg PQ, for 4 times, sacrificed) ○
- Group I (2 mg/kg PQ, for 4 times, expired) ⊕
- Group II (2 mg/kg PQ, for 5 times, sacrificed) □
- Group II (2 mg/kg PQ, for 5 times, expired) ⊠

DISCUSSION

Subcutaneous administration of paraquat dichloride (2 mg/kg), four or five times, every two days, led to death for about 70% of the monkeys treated in this experiment. All of the monkeys that expired after PQ administration had severe fibrotic lung alterations. In the research of Akahori and Oehme (1983), a total of 103 rats received the 7 mg/kg of PQ for seven or eight days. From day 8 to 16, 30% of the animals died. Approximately 53% of these rats lost bodyweight and developed pulmonary fibrosis. The remaining 17% of the rats in this study gained weight but had no discernable changes in their lungs. However, the rats who had developed pulmonary fibrosis, induced by PQ, exhibited large individual variations in the degree of pulmonary fibrosis. Also fatal pulmonary fibrosis was not apparent during the 39 day or 100 day observation periods. Griffin et al. (1979) reported that 50% of the rats who were given PQ, s.c., for five days, using doses of 1/5 LD₅₀ (5 mg/kg PQ ion/kg) died within four days after the PQ administration was completed. As their experiment consisted of a 10 day observation period after the completion of PQ administration, we have no information regarding the details of the fatal pulmonary fibrosis. In an experiment using the dog as his animal model, Akahori et al. (1985), found that producing PQ-induced fatal pulmonary fibrosis was very difficult.

In the work of Schoenberger et al (1984), lavage fluid from PQ exposed

cynomolgous monkeys contained an increased amount of the fibronectin as the fibroblast chemoattractant. They also reported that the alveolar macrophages from the PQ-exposed monkeys showed an increased fibronectin production, suggesting that the local production accounted for part of the increased amount of this glycoprotein (1984). For the plasma fibronectin levels, no significant changes were detected in our study. Rennard et al. (1981), Akiyama et al. (1983) and Hashigami et al. (1984) also reported that plasma fibronectin exhibited no variation in diffuse interstitial lung diseases.

Kucharz et al. (1982) reported that the serum free hydroxyproline concentration of humans decreased during the first few days post myocardial infarction, subsequently increased during the course of the healing process and was accompanied by an increased accumulation of collagen in the heart muscle. In the work of Fujii et al. (1981), the serum free hydroxyproline was found to be elevated in osteogenesis imperfecta, osteoporosis of the sesamoid, hypoplasia, wobbles, periostitis and bone metastasis of tumors, but it was decreased in some cases of serious disorders of the skeletal and cardiac muscles in the horse. In this study, however, the serum free hydroxyproline level was not significantly altered by exposure to PQ.

In our present study the serum ceruloplasmin levels increased significantly from day 14 to 21 (days 8 to 15 after the completion of 4 PQ doses). The report of Beretta et al. (1981) demonstrated that plasma ceruloplasmin levels always rose after a single PQ administration (p.o.) and that this was evidence of the stress condition endured by their animals. Furthermore, they stated that the increase observed in the ceruloplasmin concentrations cannot be attributed specifically to the action of PQ, as there are many other hormone dependant or stress conditions which may elicit this increase in ceruloplasmin. Divertie et al. (1982) also reported that PQ induce the acute phase reaction which, in turn, caused an increase in ceruloplasmin. However, from the results obtained in our study (the ceruloplasmin was markedly increased on day 21 after PQ administration) this increase of the ceruloplasmin cannot be explained as resulting from a stress condition, since the increase in the ceruloplasmin did not appear soon after the PQ had been administered but three weeks (21 days) later. This increase of the ceruloplasmin concentration on day 21 after the PQ injections of our monkeys correlates with a specific PQ effect, that is, the development of PQ-induced pulmonary fibrosis.

We demonstrated that paraquat-treated monkeys had elevated levels of lung hydroxyproline. Paraquat-treated rats (Griffin et al., 1979 ; Akahori et al., 1983) and dogs (Akahori et al., 1985) also experienced markedly increased levels of lung hydroxyproline. These increased levels of lung hydroxyproline suggest the inducement of pulmonary fibrosis by paraquat. In this study Nomura et al. (1986) reported that pulmonary fibrosis was not demonstrable among the four surviving monkeys from the PQ treatment group using histopathological examination. Also PQ-induced pulmonary fibrosis develops in all other Japanese monkeys in a pattern very similar to man, but is not seen in rabbits (Butler et al., 1971 ; Ilett et al., 1974)

and guinea pigs (Murray et al., 1972). Akahori et al. (1983) found large individual variations in the degree of pulmonary fibrosis induced by paraquat and fatal pulmonary fibrosis was not apparent in this previous study using rats. In another of previous experiments using dogs, we found it very difficult to induce pulmonary fibrosis, as some of the animals died from acute and/or subacute PQ toxicity (Akahori et al., 1985). In conclusion, there is a need for an experimental animal model in which paraquat-induced pulmonary fibrosis can be readily produced and in which the disease follows a course very similar to humans. Then effective treatment and antidote evaluation studies can be undertaken using these experimental animals as models of humans. After reviewing the data obtained from the serum ceruloplasmin levels, lung collagen content and histopathological examinations (Nomura et al., 1986), our data supports the concept that monkeys may prove to be a useful animal model for the study of PQ-induced pulmonary fibrosis in humans. The monkeys can provide extensive opportunities for research on both the mechanism and the treatment of PQ poisoning in man.

ACKNOWLEDGMENTS

This work was supported by the Science Research Promotion Fund of Japan Private School Promotion Foundation.

REFERENCES

- Adachi, H., Yokota, T., Fujihara, S., et al. (1978) : Two autopsy cases of paraquat poisoning. *Tohoku J. Exp. Med.*, **125**, 331–339.
- Akahori, F. and Oehme, F.W. (1983) : Inhibition of collagen synthesis as a treatment for paraquat poisoning. *Vet. Hum. Toxicol.*, **25**, 321–327.
- Akahori, F., Okinaka, T., Masaoka, T. et al. (1985) : Inhibition of collagen synthesis, dehydropyridine and paraquat-induced pulmonary fibrosis. *Proceedings of 12th Annual Meeting of Jpn. Soc. Toxicol. Sci., J. Toxicol. Sci.*, **10**, 260.
- Akiyama, S.K. and Yamada, K.M. (1983) : Fibronectin in disease. *Monograph Pathol.*, **24**, 55–96.
- Beretta, C., Fadini, L., Montesissa, C., et al., (1981) : Membrane lipid composition of subcellular fractions from lungs of rats treated with paraquat. *Pharmacol. Res Comm.*, **13**, 433–442.
- Bullivant, C.M. (1966) : Accidental poisoning by paraquat : Report of two cases in man. *Brit. Med. J.*, **1**, 1272–1273.
- Butler, C. II and Kleinerman, J. (1971) : Paraquat in the rabbit. *Brit. J. Industr. Med.*, **28**, 67–71.
- Divertie, M.B., Owen, Jr. C. A., Barham, S.S. et al. (1982) : Accumulation of radionuclide-labeled platelets and fibrinogen in paraquat-damaged rat lungs. *Am Rev. Respir. Dis.*, **125**, 574–578.

- Fitzgerald, G.R., Barnville, G., Flanagan, M., et al. (1978) : The changing pattern of Paraquat poisoning : An epidemiologic study. *J. Irish Med. Assoc.*, **71**, 103-108.
- Fletcher, K. (1972) : Paraquat poisoning. Forensic toxicology proceedings of a symposium held at the chemical defense establishment, Porton Down, June : 86-98.
- Fujii, Y., Watanabe, H., Ueda, Y., et al. (1981) : Diagnostic value of free hydroxyproline in horse serum. *Bull. Equine Res. Inst.*, **18**, 73-83.
- Griffin, M., Smith, L.L. and Wynne, J. (1979) : Changes in trans-glutaminase activity in an experimental model of pulmonary fibrosis induced by paraquat. *Br. J. Exp. Path.*, **60**, 653-661.
- Hashigami, H., Yoshikawa, K., Mizutani, K., et al. (1984) : Plasma fibronectin in various diffuse pneumonitis. *Jpn. J. Thorac. Dis.*, **22**, Supp., 157 (in Japanese).
- Heath, D. and Smith, P. (1977) : The pathology of the lung in paraquat poisoning. In *biochemical mechanism of paraquat toxicity*, 1st ed., p. 39-55. Academic Press, New York.
- Ilett, K.F., Stripp, B., Menard, R.H., et al. (1974) : Studies on the mechanism of the lung toxicity of paraquat ; Comparison of tissue distribution and some biochemical parameters in rats and rabbits. *Toxicol. Appl. Pharmacol.*, **28**, 216-226.
- Jamall, I.S., Finelli, V.N. and Que Hee, S.S. (1981) : A simple method to determine nanogram levels of 4-hydroxyproline in biological tissues. *Anal. Biochem.*, **112**, 70-75.
- Kucharz, E., Drozd, M., Cera, B. et al. (1982) : Collagen metabolism in patients with myocardial infarction. *Corvasa*, **24**, 339-344.
- Miyamoto, M., Soma, K. and Tomita, T. (1983) : Paraquat poisoning. *ICU and CCU*, **70**, 200-205 (in Japanese).
- Murray, R.E. and Gibson, J.E. (1972) : A comparative study of paraquat intoxication in rats, guinea pigs, and monkeys. *Exp. Mol. Pathol.*, **17**, 317-325.
- Naito, H., Yamashita, M., Takeshima, et al (1984) : Present status of paraquat poisoning in Japan. Abstract of AAPCC/AACT/ABMT/CAPCC Annual Scientific Meeting. *Vet. Hum. Toxicol.*, **26**, 405.
- Nomura, Y., Shiota, K., Une, Y., et al. (1986) : Paraquat-induced pulmonary fibrosis —Lung changes by paraquat in Japanese monkeys—. Abstract booklet of the **101st** Japanese Society of Veterinary Science, **68** (in Japanese).
- Ravin, H.A. (1956) : Rapid test for hepatolenticular degeneration. *Lancet*, 270 (6925), 726-727.
- Rennard, S.I., Hunninghake, G.W., Bitterman, P.B. et al. (1981) : Production of fibronectin by the human alveolar macrophage ; Mechanism for the recruitment of fibroblasts to sites of tissue injury in interstitial lung diseases. *Proc. Natl. Acad. Sci.*, **78**, 7147-7151.
- Rice, E. W. (1962) : Standardization of ceruloplasmin activity in terms of international enzyme units. *Anal. Biochem.*, **3**, 452-456.

- Schoenberger, C. I., Rennard, S. I., Bitterman, P. B., et al., (1984) : Paraquat-induced pulmonary fibrosis — Role of the alveolitis in modulating the development of fibrosis—. *Am. Rev. Respir. Dis.*, **129**, 168–173.
- Stegemann, H. (1958) : Mikrobestimmung von Hydroxyproline mit Chloramin-T und p-Dimethylaminobenzaldehyd. *Z. Physiol. Chem.*, **311**, 41–46.
- Yamauchi, J. and Kojima, T. (1982) : Review of paraquat poisoning. *Masui To Sosei*, **18**, 175–182 (in Japanese with an English summary).