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In Vivo Dual Effects of Vitamin C on Paraquat-Induced Lung Damage: Dependence on Released Metals from The Damaged Tissue

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Vitamin C, a potent antioxidant, can act as a pro-oxidant in the presence of free transition metal ions by accelerating the Fenton reaction. An *in vivo* pro-oxidant role of vitamin C has been suggested, but direct evidence for it is scant. Here, we report the dual role of vitamin C on paraquat-induced lung injury, which appears to depend on the metal ions released from damaged cells. Vitamin C (10 mg/kg) given at the time when the extensive tissue damage was in progress aggravated the oxidative damage, while it protected against the damage when given before the initiation of the damage. The extent of oxidative tissue damage was monitored by measuring the expiratory ethane, one of the hydrocarbons produced during lipid peroxidation. Deferoxamine, given intraperitoneally as a bolus dose of 50 mg/kg, completely blocked the aggravation of oxidative damage by vitamin C. Moreover, deferoxamine unmasked the antioxidant effect of vitamin C. The results show that vitamin C can either aggravate or alleviate the oxidative tissue damage depending on the presence of metal ions released from damaged cells.

Keywords: Vitamin C, pro-oxidant, paraquat, lung, Fenton reaction, deferoxamine

INTRODUCTION

Tissue damage by oxidizing free radicals is involved in various diseases including inflammation, ischemia/reperfusion and shock, diabetic complications, intoxication by exogenous toxins, and the aging process.[1] The oxidizing radicals are endogenously generated or introduced from the environment in various forms. Hydroxyl radical, the most reactive form, is generated from hydrogen peroxides via a Fenton reaction, in which free transition metal in the aqueous phase plays a critical role.[1] The Fenton reaction is applied *in vitro* to oxidize lipids and nucleic acids. It is also suspected to be involved in *in vivo* oxidative damage in various situations such as inflammation,[2] ischemia-reperfusion damage,[3,4] diabetic complications,[5] degenerative brain diseases,[6,7] iron overload diseases,[8] and cancer.[9,10]

Extracellular and intracellular defence systems protect biomolecules against oxidative damage by quenching the radicals, chelating metals from aqueous phase, and reducing, repairing, or removing damaged molecules. Generally, vitamin C is known to act as a potent antioxidant, quenching radicals and regenerating vitamin E.[1,11] On the other hand, vitamin C also accelerates the generation of hydroxyl radicals upto several hundred

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times by accelerating the redox cycling of Fe^{+++}/Fe^{++} when it exists with free transition metal ions in the aqueous phase.[12,13] Although the possibility of a pro-oxidant role of vitmina C *in vivo* in combination with transition metals has been suspected,[14] the direct evidence is scant.

It is quite possible that vitamin C acts as a pro-oxidant instead of an antioxidant once transition metal ions are released into the aqueous phase by an extensive cellular damage.[14] We probed this possibility by investigating the effect of vitamin C on paraquat-induced lung damage in rats. Paraquat inflicts lung damage after it is metabolized into radicals.[15] Our hypothesis is that vitamin C given before paraquat would protect the lung by quenching radicals as soon as they are produced. On the other hand, when vitamin C is given to ongoing tissue damage, it would aggravate the damage by interacting with free metal ions released from damaged cells to accelerate the hydroxyl radical production. Measurement of expiratory ethane was employed to assess the extent of oxidative tissue damage. This also enabled the time dependent monitoring of tissue damage.

MATERIALS AND METHODS

Chemicals

Paraquat dichloride (methyl viologen), deferoxamine, streptomycin, vitamin C (as free ascorbic acid), guanidine HCl, 1,1,3,3-tetra-ethoxypropane and thiobarbituric acid (TBA) were purchased from the Sigma Chemical Co. (St. Louis, MO, USA); ethanolamine, acetonitrile and cyclohexane from Aldrich Chemical Inc. (Milwaukee, WI, USA); 2,4-dinitrophenylhydrazine (DNPH) from Eastman Chemical Co. (Rochester, NY, USA). All other reagents were of the highest purity available.

In Vivo Monitoring of Expiratory Ethane

For the *in vivo* monitoring of expiratory ethane, the method of Lawrence and Cohen[16] was modified to concentrate the ethane content in the expiratory gas (Fig. 1). Male Sprague-Dawley rats (350 to 400g) were fasted overnight before the experiment. The tracheal cannulation was performed under anesthesia with pentobarbital sodium (40 mg/kg), and the animal was subjected to the closed ventilation. The ventilation loop was closed during the collection of ethane as indicated as the solid line in Figure 1. During the collection of ethane, carbon dioxide was trapped by KOH (10%) solution and the volume was replaced by oxygen. The reduced volume of the oxygen reservoir was again replaced by water.

After the designated duration of collection, the air was sampled from the trapping bottle and analyzed on the gas chromatography—flame ionization detector (GC-FID; Model HP5860, Hewlett- Packard) for the quantitation of ethane, using PLOT (porous layer open tubular) fused silica column (length of 50 m, inner diameter of 0.32 mm; outer diameter of 0.45 mm, liquid phase of Al_2O_3/KCl; Chrompack, Netherlands). The temperature of the injector and detector was set at 80°C and 100°C, respectively. The pure ethane (Matheson Gas Products, East Rutherford, NJ) was used for the standard chromatogram. The amount of ethane was expressed as the integrated area of the peak. To confirm the ethane peak on the gas spectrometry, the mass spectrum was obtained on the Tribrid mass spectrometer (Fison, U.K.) using the electron ionization method. The spectrum was compared to that of standard ethane.

Drug Administration

Paraquat (50 mg/kg in saline) was administered intraperitoneally. The control rats received an equivalent volume of saline. Vitamin C (10 mg/kg, as free ascorbic acid) was prepared in citrate buffer (pH7.4) to make the final volume of injection no more than 2 ml. Vitamin C was injected slowly through the femoral vein, and the plasma level of total ascorbate (ascorbic acid plus dehydroascorbic

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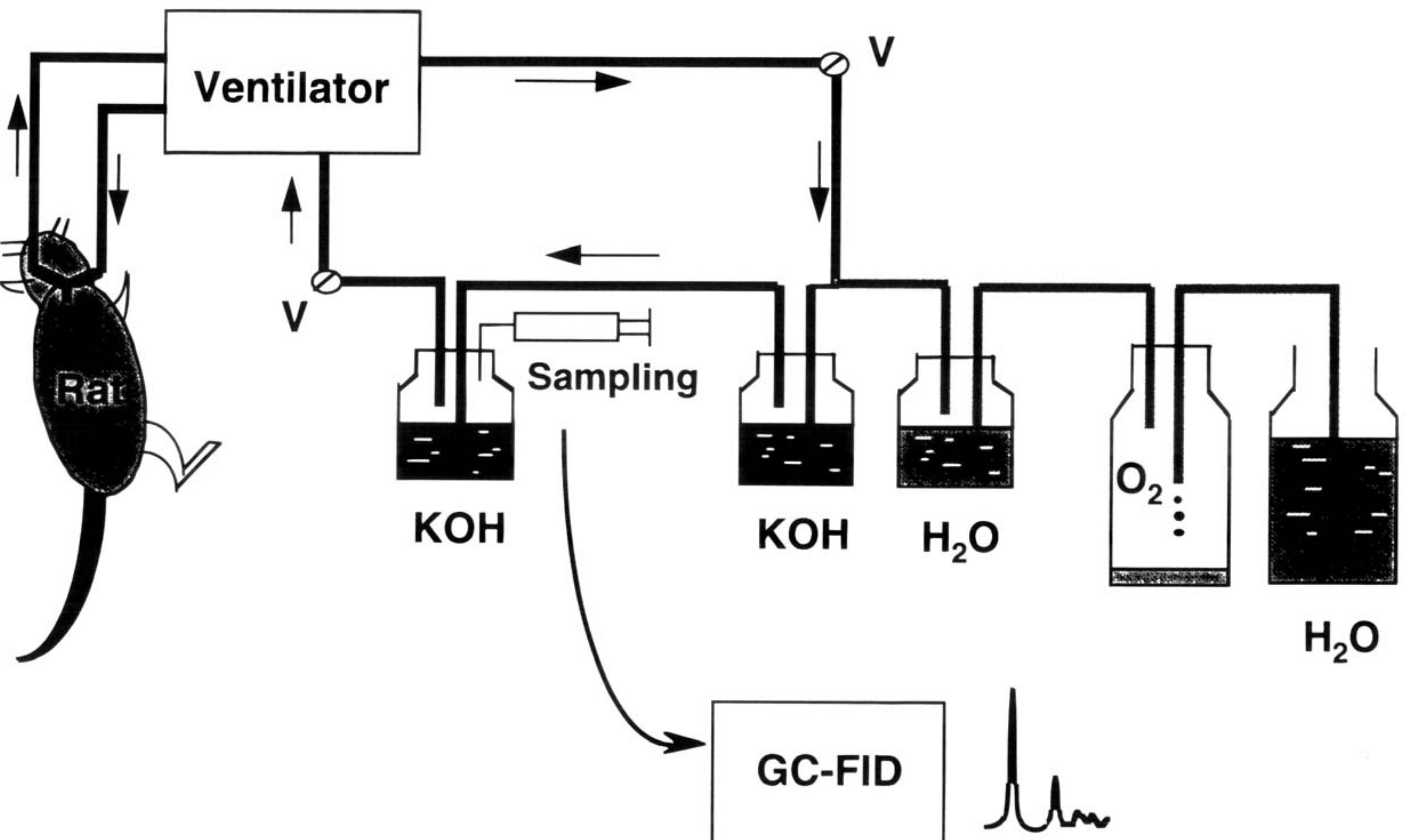

FIGURE 1 Set-up for *in vivo* collection of expiratory gas. During the collection, the three-way valves (V) were positioned to make the ventilation through the closed loop (solid line). Air space in two bottles of 10% KOH was less than 50 ml each.

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acid) was measured before (basal), 30 minutes, 60 minutes and 120 minutes after the injection according to the method by Lykkesfeldt *et al.*[17] Vitamin C injection did not change arterial pH. Deferoxamine (50 mg/kg) in saline, was intraperitoneally administered.

Morphological Examinations

After the ethane collection, the lung was perfused with heparinized saline to wash out blood, excised, fixed in 10% formalin solution, and paraffin blocks were prepared from the fixed tissue. Tissue blocks were cut, stained with hematoxylin-eosin, and observed under the light microscope.

Assays of Oxidized Proteins and Lipids

The lungs were excised following complete removal of RBC by perfusing with ice-cold saline after the ethane collection. The excised lungs were rinsed with cold saline, frozen rapidly in liquid nitrogen, ground with mortar and pestle, and finally homogenized with a glass-Teflon homogenizer on ice. The homogenate was passed through gauze to remove tissue debris.

Protein oxidation was assessed by the determination of protein carbonyl group content with DNPH incorporation technique.[18] Briefly, 2 equal aliquots of the supernatant fraction of tissue homogenate were precipitated with trichloroacetic acid (TCA). One aliquot was reacted with 10 mM DNPH in 2 N HCl for an hour in room temperature and the other one with 2 N HCl only. The protein was precipitated again with TCA and the free DNPH was removed by washing with ethanol:ethylacetate (1:1, v/v). After the protein was dissolved in 6 M guanidine HCl solution, the absorbance at 365 nm was measured. The concentration of carbonyl groups was calculated with the molar absorptivity of $2.2 \times 10^4\ M^{-1}cm^{-1}$ for the aliphatic DNPH derivatives, and expressed as nmol/mg protein. The protein content was determined by the method of Bradford.[19]

The oxidized lipid was quantified by measuring the malondialdehyde (MDA)[20,21] and conjugated dienes (CD).[22,23] In order to measure the content of conjugated dienes, the total lipid was extracted from the tissue homogenate with chloroform/methanol(1:1, v/v) and chloroform/methanol/water (86:14:1). The lipid extract was dried under pure nitrogen (>99.999%), dissolved in cyclohexane, and the absorbance was measured at 234 nm. The concentration of CD was calculated from the molar extinction coefficient of $2.7 \times 10^4\ M^{-1}cm^{-1}$. The weight of the total lipid extract was measured after the complete drying, and the content of CD was expressed as nmol/mg lipid. MDA content was measured with high performance liquid chromatography (HPLC) following TBA reaction. Briefly, the tissue homogenate was reacted with 1% TBA in 5% TCA at 95°C for 30 min. After extraction with ethyl acetate containing diethylether, the TBA-MDA complex substance was separated using an ODS c-18 column (μBondapak, Waters) with the mobile phase of 0.1% ethanolamine/acetonitrile (2:1, v/v), monitoring the absorbance at 592 nm. The concentration of MDA was calculated on the standard curve using 1,1,3,3-tetra-ethoxypropane and expressed as nmol/mg protein.

Statistical Analysis

Values are mean ± standard error. The influence of each intervention on ethane and biochemical measurements was assayed by one-way analysis of variance using the Stat View IV (Abacus Concepts, USA) statistical program. Difference in plasma levels of vitamin C between groups was tested by ANOVA followed by Fisher's post hoc test.

RESULTS

In Vivo Monitoring of Expiratory Ethane Produced from Paraquat-Treated Rats

Paraquat, 50 mg/kg, did not cause a significant mortality over 24 hour period (only 1 out of 10

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rats died) although signs of respiratory distress (irregular and forced respiration) were apparent. Vitamin C, 10 mg/kg, given either before or after paraquat, did not affect the mortality (0 and 1 out of 10, respectively) or the respiratory distress.

The expiratory ethane that is collected after paraquat treatment was quantified on the GC-FID. The ethane peak was identified by comparing the elution time against standard ethane (Figs. 2A and B), and this was confirmed by the mass spectrometry (Figs. 2C and D). In the spectrum of the sample, the huge m/z (28) value of nitrogen overlaped the m/z 28 peak of ethane, which dwarfed the relative peaks of other m/z values. Oxygen (m/z of 32) and water (m/z of 18) peaks were also visible. Other than that, the sample spectrum matches that of standard ethane.

The amount of ethane in the expired air significantly increased during the first hour after the paraquat injection (Fig. 3). The ethane production increased further during the second hour, suggesting that the tissue damage was progressing extensively. The ethane production decreased slowly after then.

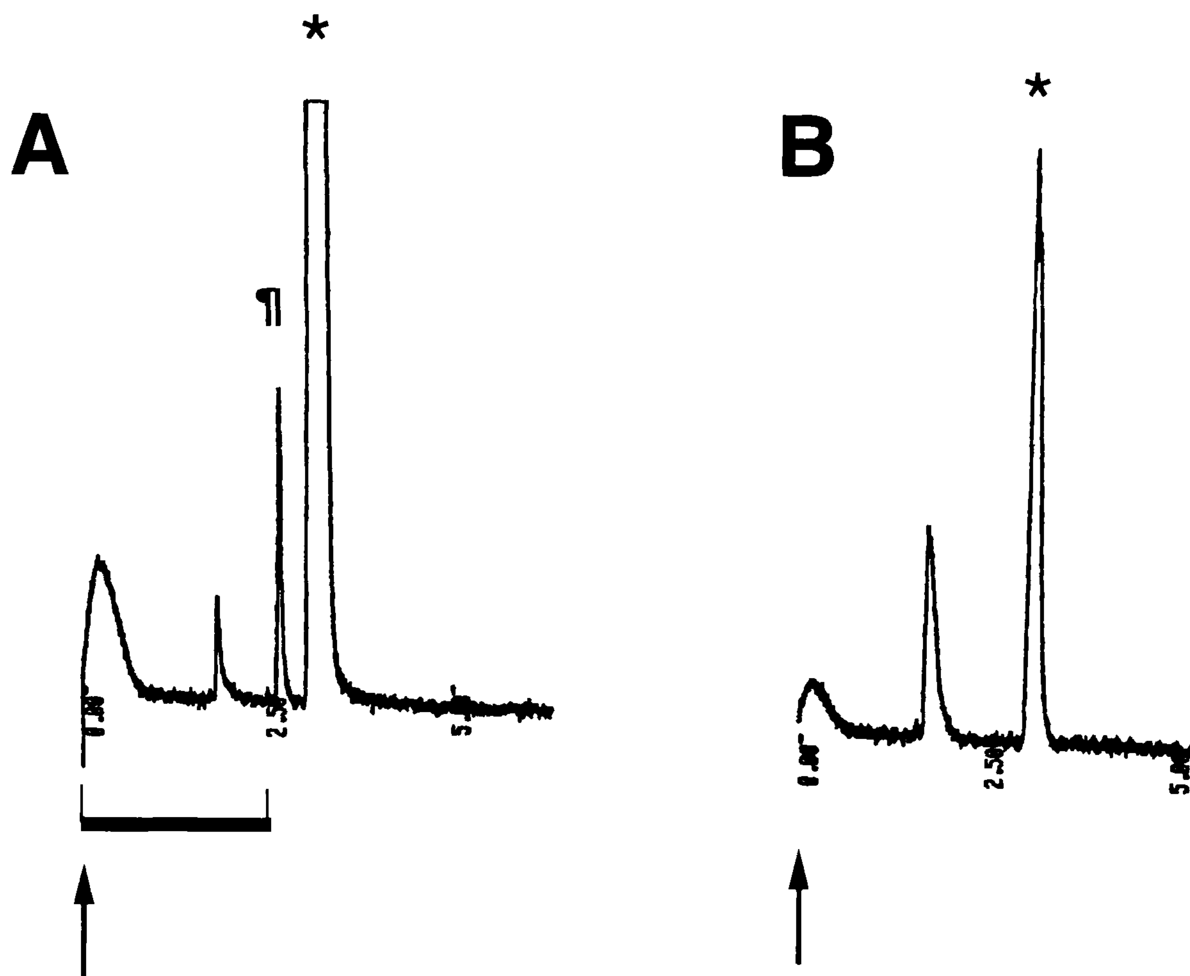

FIGURE 2 GC-FID detection of ethane (*) from standard gas **(A)** and from expiratory gas from a paraquat-treated rat **(B)**. Air samples were injected on GC at the point indicated by arrows. The bar describes the time span of 2.5 minutes. A small peak of methane (¶) contaminating the standard gas is visible before the ethane peak on panel A. Mass spectrum of ethane peak from sample **(D)** is compared to that of standard ethane **(C)**. Big M/Z peaks of nitrogen (at 14 and 28), oxygen (at 16 and 32), and water (at 18) are visible on the sample spectrum. The profile of other peaks is of ethane.

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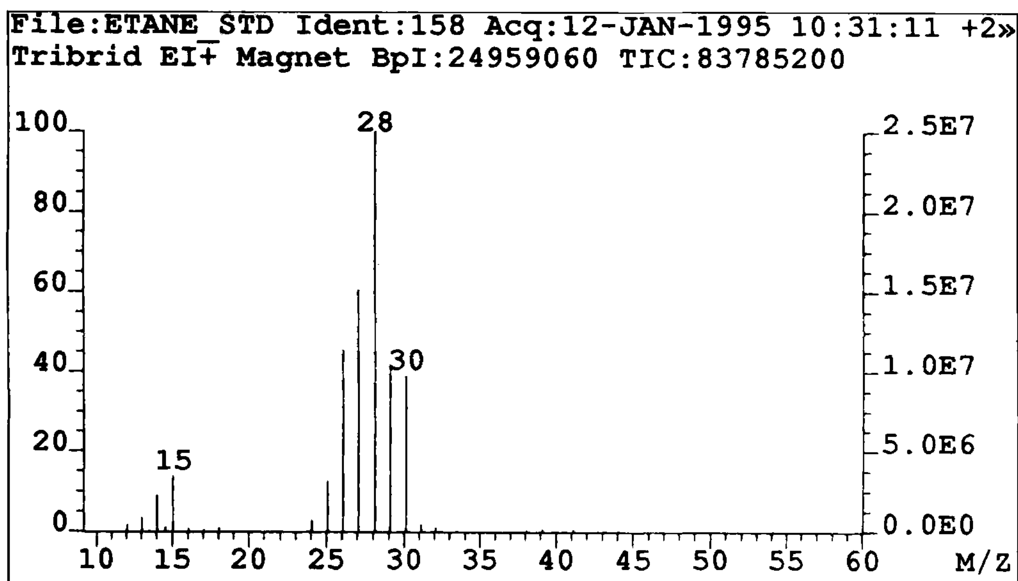
C



D

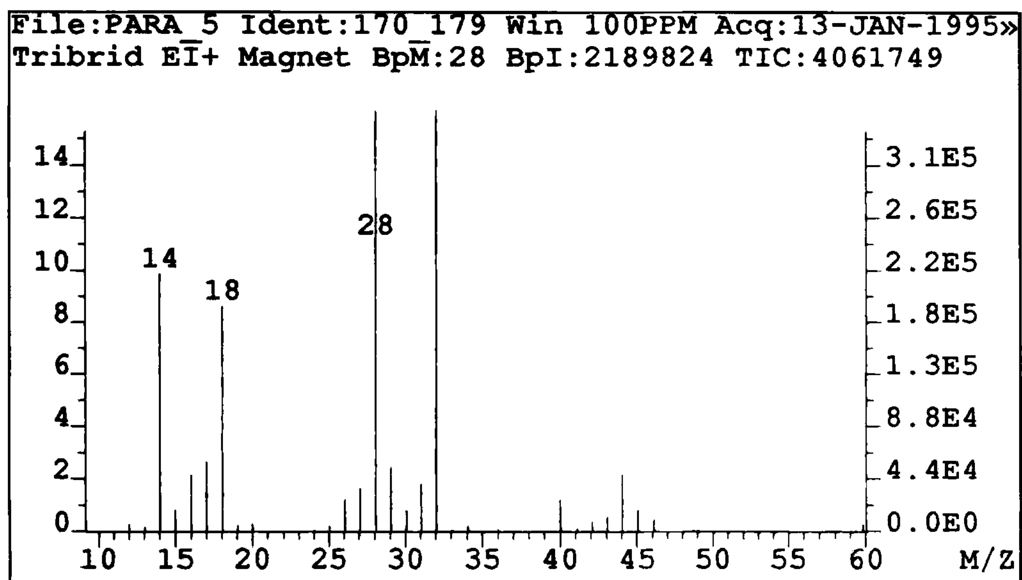


FIGURE 2 (*Continued*)

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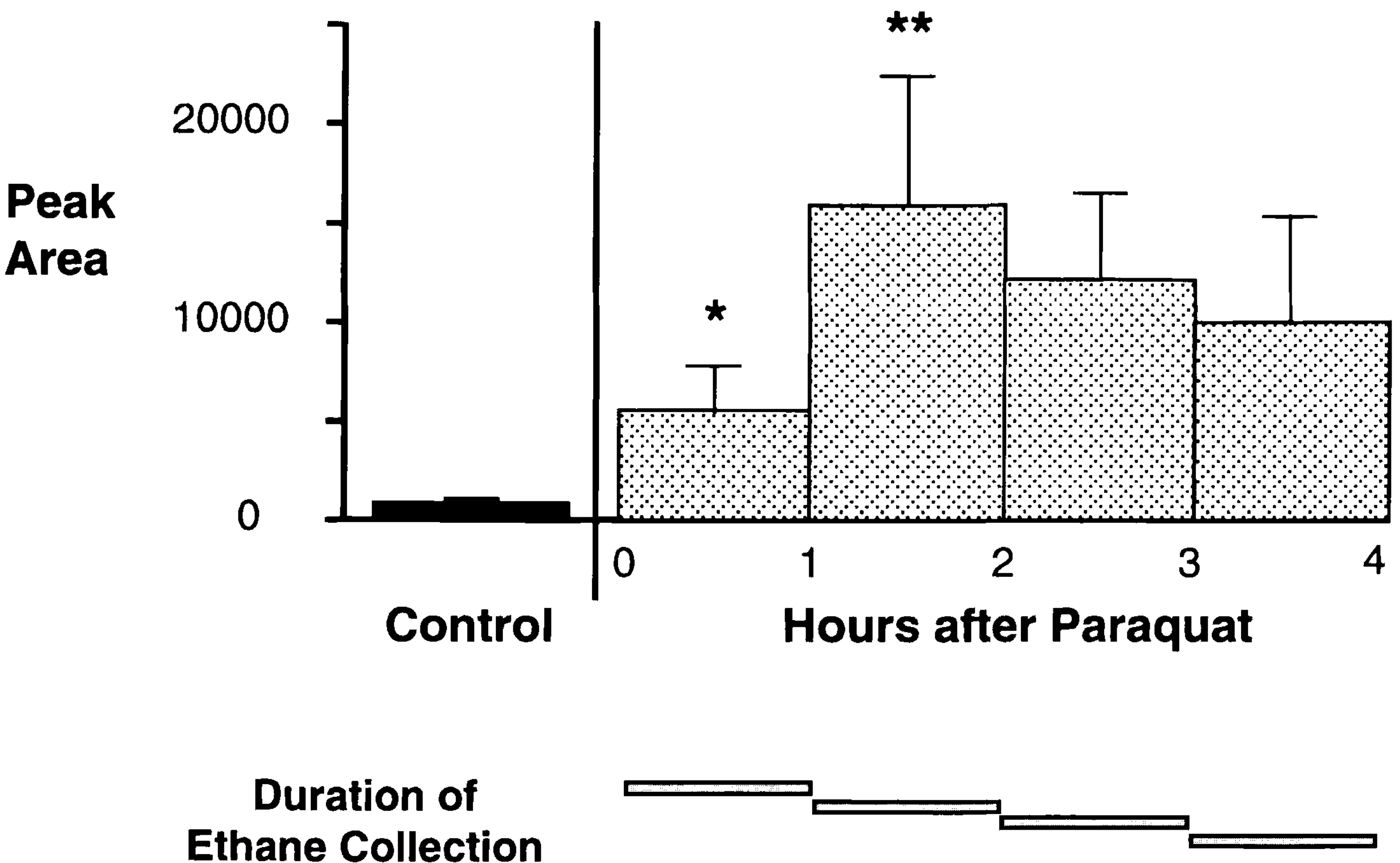

FIGURE 3 Time course of ethane production in paraquat-treated rats. The rats were subjected to the closed-loop ventilation for an hour, starting from 0, 1, 2, and 3 hours after paraquat injection (50 mg/kg). After the collection, 5 ml of air from the first bottle of KOH was sampled and analyzed on the GC-FID. The quantity of ethane is expressed as the integrated area of the ethan peak. ($n = 5$ each, *: $p < 0.01$ vs. Control, **: $p < 0.01$ vs. 0–1 hr collection)

Paraquat-Induced Protein and Lipid Oxidation

The lung tissue was collected 2 hours after the paraquat injection and assayed for protein and lipid oxidation (Table I). Paraquat injection significantly increased the DNPH incorporation into the carbonyl groups of the tissue protein, which suggested an increase in protein oxidation. Two indices of lipid peroxidation, MDA and CD, also were increased by paraquat, although the statistical significance of the former was marginal.

The Effects of Vitamin C and Deferoxamine on Paraquat-Induced Ethane Production

The effect of vitamin C on paraquat-induced ethane production appeared to depend on the timing of administration (Fig. 4). Intravenous administration of 10 mg/kg vitamin C 5 minutes before the paraquat injection ('pretreatment of vitamin C') reduced ethane production by 60.2 ± 5.4%. On the other hand, vitamin C given 1 hour after the paraquat injection ('post-treatment of vitamin C') increased the paraquat-induced ethane production by 44.2 ± 3.5%.

Plasma levels of total ascorbic acid increased immediately after the injection of vitamin C and stayed significantly higher than the basal level throughout the experimental period (Table II). There was no difference in the total ascorbate level between the two groups of paraquat-treated rats, the 'pre-treatment' and 'post-treatment' of vitamin C. The profile of ascorbate level in control rats after vitamin C injection showed no difference except 30 minute level, which was a little higher than paraquat-treated groups. Paraquat alone did not change the basal plasma ascorbate over two hour period of the experiment (μM at 0, 30, 60 and 120 minutes after i.v. injection: 30.86 ± 0.18, 32.65 ± 1.68, 31.06 ± 1.19, and 33.61 ± 1.03 from 5 animals).

TABLE I Biochemical indices of peroxidation of the lung tissue from control and paraquat-treated rats

	Control	Paraquat
DNPH nmol/mg protein	1.58 ± 0.10	2.09 ± 0.10*
MDA nmol/mg protein	1.41 ± 0.15	1.91 ± 0.08**
CD nmol/mg lipid	22.02 ± 2.52	29.29 ± 3.23*

(n = 5 each, *: $p < 0.01$ vs. Control, **: $p = 0.08$)

Effects of Deferoxamine

Deferoxamine (50 mg/kg), injected 10 minutes before the paraquat administration, decreased the paraquat-induced ethane production by 29.9 ± 5.9% ($p < 0.01$, +DFX + paraquat vs. –DFX + paraquat; Fig. 5). This effect of deferoxamine was more prominent in rats with paraquat plus post-treatment of vitamin C, reducing ethane production by more than 80% to the level significantly lower than that in rats with paraquat only (26.1 ± 16.6% vs. 70.1 ± 5.9%, $p < 0.05$).

Morphological Observation

The lungs excised 2 hours after the paraquat treatment showed many small hemorrhagic spots on gross morphology. Under the microscope, paraquat caused cell infiltration of alveolar septa leading to thickening and exudate in some alveolar spaces (Fig. 6B). While 'pretreatment of vitamin C' prevented these pathological changes (Fig. 6C), 'post-treatment of vitamin C' aggravated the damage (Fig. 6D). Deferoxamine prevented the pathological changes induced by paraquat plus 'post-treatment of vitamin C,' preserving almost the normal morphology (Fig. 6E).

DISCUSSION

In the current study, we provide evidence of *in vivo* pro-oxidant action of vitamin C. Free metals must be present at the time of vitamin C introduction for this, because deferoxamine blocked the pro-oxidant effect of vitamin C (Fig. 5). Vitamin C was a pro-oxidant when given after the paraquat injection, while it was an antioxidant when given before paraquat. This suggests

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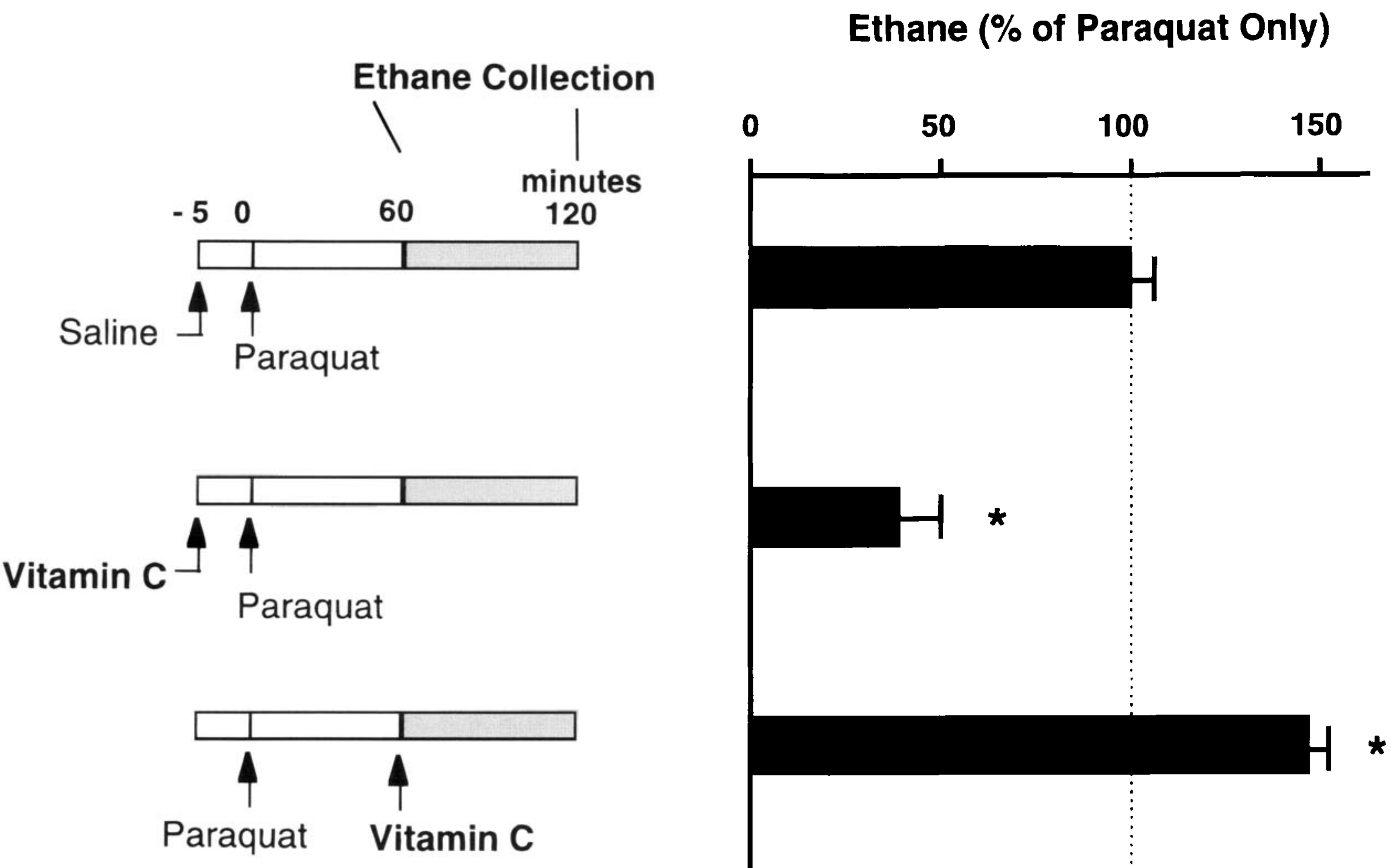

FIGURE 4 Effects of vitamin C on paraquat-induced ethane production. Columns on the left depict time schedule of the events during the experiments. The ethane production is expressed as the percentage of that by paraquat only. Upper schedule: paraquat only. Middle schedule: pretreatment of vitamin C and paraquat. Lower schedule: paraquat and post-treatment of vitamin C. ($n = 5$ each, *: $p < 0.01$ vs. paraquat only)

TABLE II Plasma levels of total ascorbate in μM after i.v. injection of 10 mg/kg of vitamin C. Control: vitamin C injection without paraquat treatment, Pre-VitC: vitamin C injection 5 minutes before paraquat, Post-VitC: vitamin C injection 1 hour after paraquat.

Minutes after vitamin C injection	0	30	60	120
Control	31.35 ± 0.11	108.58 ± 7.69	73.90 ± 4.57	60.49 ± 1.95
Pre-VitC	–	80.34 ± 1.49*	68.11 ± 3.68	52.98 ± 2.39
Post-VitC	–	77.00 ± 1.88*	70.05 ± 3.35	50.50 ± 7.96

($n = 5$ each, *: $p < 0.05$ vs. Control)

that the source of metal ions in the current study is the cells undergoing damage or dying.

The damage of biological molecules by the mixture of vitamin C with transition metal ions has been shown mostly in *in vitro* conditions, including a recent report on human brain tissue.[24] Recently, the possibility of *in vivo* pro-oxidant action of vitamin C was raised by Halliwell,[14] reviewing toxic effects of megadoses of vitamin C and anecdotal reports of damage by vitamin C in various clinical conditions. However, direct evidence of *in vivo* prooxidant effects of vitamin C is still scant and contradictory.

As Halliwell pointed out, the key question about the vitamin C-induced oxidation is the availability of transition metal ions. The free transition metals in the body fluid can be hardly measured *in vivo*, for they are bound by proteins and other biomolecules. However, even trace amounts of transition metals can serve as catalysts for oxidative processes.[12] Most studies evidencing the involvement of transition metals in oxidative damage are indirect, employing metal chelators to reduce the damage.[2,3,4,6,7] It could be the on-going tissue damage at the time of vitamin C presence that increases the availability of transition metal directing the action of vitamin C to the pro-oxidation. We tried to differentiate the protective and the deleterious effects of vitamin C on the same type of *in vivo* oxidative tissue damage, simply by the different timing of vitamin C administration.

We chose the lung as the target tissue to study the dual effects of vitamin C *in vivo*, because it is one of the most vulnerable organs to oxidative injuries,[25] and has a wide range of intracelluar and extracellular antioxidant defences.[26,27] Several metal binding proteins are present in lung lining fluid including transferrin, ceruloplasmin and albumin.[1,11] In addition to these, the pulmonary epithelium also secretes an additional metal binding protein, lactoferrin.[27]

In our study, the rate of tissue damage is assessed by monitoring the expiratory ethane. Volatile hydrocarbons such as ethane and pentane are produced from the damaged tissue, and reflect the extent of peroxidized unsaturated fatty acids.[28,29] Ethane is relatively resistant to re-uptake and metabolism by the tissue, and can be monitored from the expiratory gas.[16,30] The current method illustrated in Figure 1 maximized the efficiency of ethane concentration, enabling time-dependent monitoring. Paraquat preferentially damages the lung, for it is concentrated by the lung and metabolized to oxidizing radicals by P450-associated enzymes before damaging the tissue.[15,31] The lung is considered as the major source of ethane with this dose of paraquat, although other sources including the liver also exist.[32] Two hours after the paraquat administration, the morphological changes of the lung tissue (Fig. 6B) and the biochemical parameters of protein and lipid peroxidation (Table I) were evident, verifying the expired ethane as a reliable index of oxidative damage. The success of our short-time monitoring of ethane from the expiratory gas depended on the closed ventilation which was directly connected to the trachea. This procedure drastically reduced the trapping volume and

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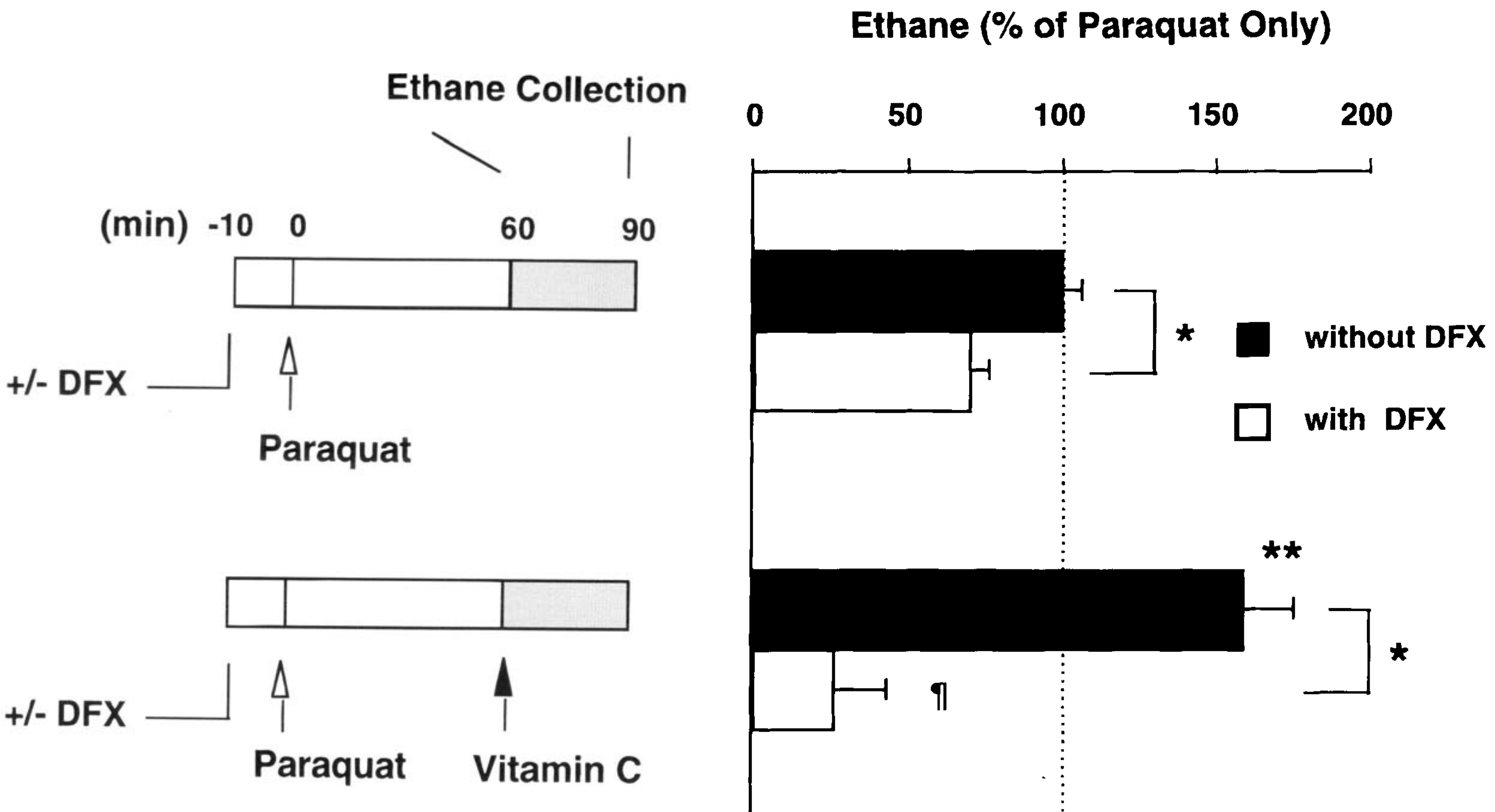

FIGURE 5 Effect of deferoxamine on expiratory ethane. Upper schedule: paraquat with/without deferoxamine (DFX; 10 mg/kg in saline). Lower schedule: paraquat plus post-treatment of vitamin C with/without deferoxamine (n = 5 each, *: $p < 0.01$ between with and without deferoxamine, **: $p < 0.01$ vs. paraquat only without deferoxamine. ¶: $p < 0.05$ vs. deroxamine only on paraquat)

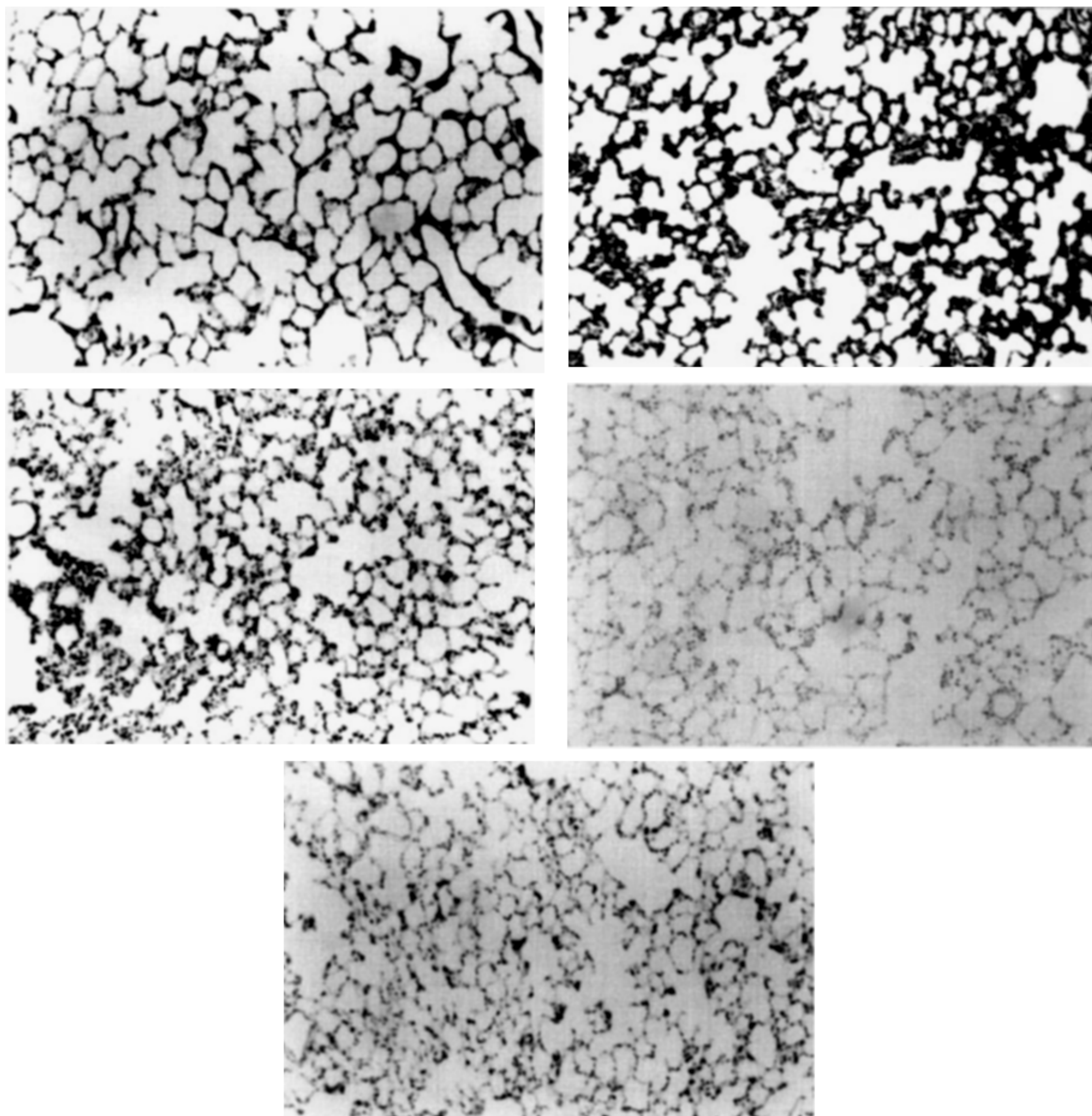

FIGURE 6 Morphology of lung tissue stained with hematoxylin-eosin. **A:** control, **B:** paraquat only, **C:** pretreatment of vitamin C (10 mg/kg) plus paraquat, **D:** paraquat plus post-treatment of vitamin C (10 mg/kg), **E:** deferoxamine (50 mg/kg) on paraquat plus post-treatment of vitamin C (10 mg/kg). Magnification: × 100.

eliminated the contamination from sources other than the expiratory gas which might be incurred with the previous whole animal ventilation.[16] This might contribute to the failure in the previous attempts to observe the ethane increase with paraquat treatment,[33] in addition to the different dose of paraquat. The current dose of paraquat (50 mg/kg) apparently caused significant tissue damage, although it was below the LD50 in rats (125 mg/kg, Merck Index, 12th ed.).

As we identified, by expiratory ethane measurement, the time that tissue damage was extensive,

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we tested the differential effects of vitamin C depending on the time of administration (Fig. 4). First, vitamin C (10 mg/kg), given before the paraquat injection ('pretreatment of vitamin C'), decreased the ethane production by more than half. Vitamin C apparently quenched radicals produced from paraquat before they attacked biomolecules. The protective role of the pretreatment of vitamin C was also confirmed morphologically (Fig. 6C). On the other hand, vitamin C, administered an hour after the paraquat injection at which the ethane production was maximum ('post-treatment of vitamin C'), aggravated the oxidative damage as evidenced by the further increase of ethane production. The morphological observation confirmed the aggravation of the damage (Fig. 6D). The higher dose of vitamin C (100 mg/kg) worsened the pathological changes with almost complete destruction of alveolar structure (data not shown). The results imply that the aggravation of oxidative damage by vitamin C requires the release of transition metals from damaged cells, as suggested recently.[12,14] The effects of single dose of vitamin C appeared to be transient, not affecting 24 hour mortality or morbidity, although it was not verified biochemically or morphologically in the current study. Effects of repeated administration of vitamin C over extended period of time need to be studied.

To confirm the role of free transition metals in the aggravation of oxidative damage by the 'post-treatment of vitamin C', we examined the effect of deferoxamine (Fig. 5). Injection of deferoxamine (50 mg/kg) 10 minutes before paraquat administration showed a clear protection against paraquat injury as evidenced by the decrease in ethane production (Figure 5) and lung morphology (figure not shown). This dose of deferoxamine also prevented the aggravation of paraquat injury by vitamin C as evidenced by reduction of ethane production (Fig. 5) and by prevention of morphological changes (Fig. 6E). Moreover, deferoxamine unmasked the antioxidant action of vitamin C: the combination of vitamin C and deferoxamine decreased the paraquat- induced ethane production below the level of deferoxamine only without vitamin C (Fig. 5). Unlike some chelators such as EDTA that can promote radical production when they are present in proper ratio to free iron in aqueous environment, deferoxamine binds very tightly to ferric iron and totally inactivates iron-mediated radical production in most cases.[34] However, the *in vivo* antioxidant effect of deferoxamine appears to be variable with various radical insults.[34–39] For example, intravenous infusion of deferoxamine has been reported to reduce the paraquat mortality in rats over the course of several days,[35] while bolus injections of deferoxamine several times a day did not improve the mortality.[36] The cause of the difference between the two studies is not clear, although the differences in dose, route, and schedule of administration can be suspected (100 to 200 mg/kg/24 h, given continuous intravenous infusion: 1,350 to 1,560 mg/kg/ 24 h, given as several subcutaneous bolus injections). Higher doses of deferoxamine do not necessarily present stronger antioxidant effects,[35] and extreme doses can even be pro-oxidant, both *in vitro* and *in vivo*.[38,39] In our study, 50 mg/kg of deferoxamine, given as a single bolus intraperitoneal injection 10 minutes before the paraquat, clearly protected the lung against the oxidative damage, which was observed in a couple of hours.

In conclusion, the present study provides direct evidence for the pro-oxidant role of vitamin C *in vivo*. The pro-oxidant effect of vitamin C appears to depend on transition metals as suggested by previous works. The source of transition metals in the current study is suspected to be the damaged tissue. The result implies that vitamin C can be deleterious in oxidative injuries when the tissue damage is already in progress and extensive enough to release significant amount of free transition metals from the sequestered compartments, while vitamin C is a potent antioxidant when it is introduced prior to that situation or when the free metals are eliminated.

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