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Vitamin E Affects Lung Biochemical and Morphologic Response to Hyperoxia in the Newborn Rabbit

DAVID F. WENDER, GUNILLA E. THULIN, G. J. WALKER SMITH, AND JOSEPH B. WARSHAW[41]

Division of Perinatal Medicine, Departments of Pediatrics and Obstetrics and Gynecology, and Department of Pathology, Yale University School of Medicine, New Haven, Connecticut, USA

Summary

The effects of parenteral vitamin E treatment on aspects of the pulmonary biochemical and morphologic response to 100% oxygen were studied in newborn rabbits manifesting chemical evidence of vitamin E deficiency. Pups treated with 2 mg/100 g body weight increased serum vitamin E levels from 0.39 to 2.17 mg/dl by 72 hr and lung tissue vitamin E content from 3.52 to 17 mg/mg wet weight of lung. *In vitro* lipid peroxidation in lung homoginates of animals in 100% oxygen for 72 hr was inhibited by approximately 80% in animals receiving 100% oxygen plus vitamin E. Hyperoxia-induced increases in the pulmonary antioxidant enzymes, superoxide dismutase, glutathione peroxidase, and glutathione reductase were diminished by vitamin E administration. Lungs from vitamin E-treated animals did not show the early lung epithelial injury seen in animals exposed to 100% oxygen but not treated with vitamin E. Mophometric analysis of lungs of animals in room air for 72 hr showed 81.6% of lung to be normal as compared with 43.3% normal lung in the group maintained in 100% oxygen for 72 hr. In the group treated with oxygen plus vitamin E, the lungs were similar to room air controls (82.6% normal). This study thus provides further evidence for a direct antioxident affect of vitamin E in lung.

Speculation

These findings provide further evidence of antioxidant protection of lung by vitamin E and suggest that the antioxidant effect of vitamin E may involve an inactivation of the probable stimulus for antioxidant enzyme induction, oxygen-free radicals.

Although oxygen is essential for survival of aerobic organisms, in excess, it can produce toxic effects which result in tissue injury. The lung is particularly vulnerable to the toxic effects of oxygen because the pulmonary epithelium may be exposed directly to high concentrations of oxygen. In the human neonate, bronchopulmonary dysplasia (BPD) has been linked to prolonged oxygen therapy for neonatal hyaline membrane disease (HMD), and although the etiology of BPD is most likely multifactorial, direct lung parenchymal injury by oxygen is postulated to be a major etiologic factor (6, 24, 26). Concern for the frequency with which BPD complicates recovery from HMD has prompted extensive research into methods of preventing or counteracting the toxic effects of oxygen on neonatal lung (38).

A preliminary clinical study from our group showed that administration of the antioxidant *dl*-α-tocopherol (vitamin E) ameliorated the course of BPD in infants recovering from HMD, suggesting that neonatal lung antioxidant protection could be augmented by vitamin E treatment (7). To further test this hypothesis, we have examined the influence of vitamin E treatment on the biochemical and morphologic response to hyperoxia in the newborn rabbit.

MATERIALS AND METHODS

New Zealand White rabbit pups were delivered by caesarean section one day before term and were randomized by weight and litter into three equal groups. All groups were maintained in incubators at 32°C and were fed a modification of the diet described by Aprille and Rulfs (1) consisting of 15 g egg albumin, 2 g lactose, and 8 g corn oil diluted with water to a total volume of 100 ml. The pups were fed this synthetic formula once a day via sialastic tubing inserted into the stomach, each pup receiving volumes of 8% of body weight on day one, 10% on day two, and 12% on day three. Group I was maintained in room air, whereas groups II and III were maintained in 100% oxygen as periodically confirmed by Beckman oxygen analyzer. Animals in group III received vitamin E (free alcohol; Hoffmann-LaRoche, Nutley, N.J.), 2 mg/100 g/day intramuscularly. In studies of lung histology, vitamin E dose was increased to 6 mg/100 g/day.

Pups were sacrificed by decapitation at birth and at 24-hr intervals up to 72 hr. Blood from two pups was collected in heparinized tubes, and the plasma was retained and frozen for vitamin E determinations. Before removal of the lungs, the thoracic cavity was exposed ventrally, and the left ventricle was incised. The pulmonary artery was cannulated and perfused with 10 cc of 0.9% saline solution to remove blood from the pulmonary vascular bed. Lung tissue from two pups was pooled and homogenized in 0.05 potassium phosphate buffer (pH 7.8) at a 1:10 w/v dilution using a Brinkman Polytron tissue homogenizer. A cytosol fraction was prepared by centrifuging the crude homogenate at 700 × *g* for 10 min to remove nuclei and debris, at 8000 × *g* for 10 min to remove mitochondria, and then at 100,000 × *g* for 60 min. Aliquots of the crude homogenate and cytosol fractions were frozen for later assays.

Plasma vitamin E was determined using a modification of the technique of Fabianek *et al.* (8). Lung tissue vitamin E content was determined using the method of Taylor *et al.* (34). Stability of red blood cells to hydrogen peroxide (H_2O_2) hemolysis was assessed according to the method of Horwitt *et al.* (17). Superoxide dismutase (EC 1.15.1.1) activity was determined on lung homogenates by measuring the inhibition of the autoxidation of epinephrine (22). Glutathione peroxidase (EC 1.11.1.9) activity in the lung cytosol fractions was determined as described by Paglia and Valentine (25). Glutathione reductase (EC 1.6.4.2) activity in homogenates was determined by the method of Horn (15). *In vitro* lipid peroxide formation by lung tissue was assessed using a modification of the 2-thiobarbituric acid method to quantify the production of malonyldialdehyde (29). Protein concentrations were determined on lung homogenates and cytosol fractions by the method of Lowry *et al.* (20). After routine fixation, embedding, and sectioning, lung sections were stained with hematoxylin and eosin for histologic examination. Specimens were compared qualitatively by an unbiased observer who then quantitated the extent of tissue abnormality for each specimen using the morphometric point-counting technique of Weibel (35).

Before statistical analysis, arcsin transformations ($x' = 2 \arcsin \sqrt{x}$) were performed on the H_2O_2 hemolysis data and morphometry data to normalize the binomial nature of these proportional measures (37). Because each experiment had been conducted on littermates, repeated-measures analysis of variance (ANOVA) was then applied in the statistical analysis of these transformed proportions and all nonporportional data. Preliminary omnibus analyses revealed significant treatment effects for every variable. Based on these initial analyses, separate repeated-measures ANOVAs were conducted on 24-, 48-, and 72-hr data for each variable. Newman-Keuls post-hoc analyses (37) were then performed on those repeated measures ANOVAs which were significant.

RESULTS

BIOCHEMISTRY

The mean plasma vitamin E level was 0.39 mg/dl at birth. As shown in Table 1, the mean plasma concentration of vitamin E in the room air and 100% oxygen groups increased only about 2-fold by 72 hr and did not differ from each other significantly. At a daily vitamin E dose of 2 mg/100 g, the mean plasma vitamin E level increased over 72 hr to 2.17 mg/dl and was significantly higher than the 72-hr results for the two other experimental groups. Animals treated with 6 mg/100 g/day had a mean plasma vitamin E level of 9.04 mg/dl at 72 hr. Tissue levels of vitamin E in lung during the study period are shown in Table 2. At 72 hr, mean vitamin E content of lung of the group in 100% oxygen treated with vitamin E (2 mg/100 g/day) was 17 μg/mg wet weight as compared with approximately 4 μg/mg wet weight in both the room air and 100% oxygen groups.

Results for the red blood cell H_2O_2 hemolysis test are presented in Table 3. For both the room air and 100% O_2 groups, percentage of red cell hemolysis increased during the course of the experiment. However, for the oxygen-exposed group treated with vitamin E, a marked resistance to H_2O_2-induced hemolysis developed with cumulative vitamin E treatment.

In vitro lipid peroxide formation was assessed in lung homogenate from animals maintained in 100% oxygen with or without vitamin E treatment. As shown in Table 4, 72 hr of oxygen exposure resulted in increased lipid peroxide formation in animals not treated with vitamin E. However, in vitamin E-treated animals, lipid peroxide formation fell with cumulative vitamin E treatment so that by 72 hr, lipid peroxide formation had been inhibited by approximately 80%.

Results for lung homogenate superoxide dismutase activity are presented in Table 5. During the course of the experiment, a gradual increase in superoxide activity was observed in animals exposed to 100% oxygen without vitamin E treatment. In contrast, animals treated with vitamin E demonstrated much smaller increases in superoxide dismutase activity in response to oxygen. Results for that group did not differ significantly from room air

Table 1. *The influence of vitamin E on the plasma vitamin E level*

	Plasma vitamin E level (mg/dl)		
Age	Room air	100% oxygen	100% oxygen + vitamin E
Birth	0.39 ± 0.05[1]		
24 hr	0.29 ± 0.08	0.58 ± 0.16	2.06 ± 0.48[2]
48 hr	0.46 ± 0.10	0.46 ± 0.13	3.01 ± 0.51[2]
72 hr	0.61 ± 0.08	0.82 ± 0.12	2.17 ± 0.40[2]

[1] Mean ± S.E. of six experiments. Vitamin E dose was 2 mg/100 g/day.

[2] Statistically significant difference from room air and 100% oxygen groups ($P < 0.01$). Values for the room air group were not significantly different from those of the 100% oxygen group. Statistical significance was determined by repeated measures analysis of variance with Newman-Keuls post-hoc test.

Table 2. *The influence of vitamin E on lung tissue vitamin E content*

	Lung vitamin E content (μg/mg wet wt)		
Age	Room air	100% oxygen	100% oxygen + vitamin E
Birth	3.52 ± 0.33[1]		
24 hr	4.36 ± 0.54	4.12 ± 0.26	9.44 ± 0.67[2]
48 hr	3.76 ± 0.34	3.52 ± 0.22	10.36 ± 1.27[2]
72 hr	4.32 ± 0.26	3.96 ± 0.25	17.04 ± 0.86[2]

[1] Mean ± S.E. of five experiments. Experimental conditions and statistical analysis otherwise were as described in Table 1.

[2] Statistically significant difference from both the room air and 100% oxygen groups ($P < 0.01$). Values for the room air group did not differ significantly from those of 100% oxygen group.

Table 3. *The influence of vitamin E on erythrocyte sensitivity to H_2O_2 hemolysis*

	% erythrocytes hemolyzed		
Age	Room air	100% oxygen	100% oxygen + vitamin E
Birth	30.8 ± 10.9[1]		
24 hr	55.5 ± 12.4	62.1 ± 10.6	15.6 ± 5.5[2]
48 hr	47.5 ± 13.5	73.4 ± 7.5	5.4 ± 1.1[2]
72 hr	71.9 ± 9.9	80.1 ± 8.5	3.7 ± 0.4[2]

[1] Mean ± S.E. Experimental conditions and statistical analysis were as described in Table 1.

[2] Statistically significant difference from both the room air and 100% oxygen groups ($P < 0.01$). Values for the room air groups differed significantly from the 100% oxygen group at 48 hr only ($P = 0.05$).

Table 4. *The influence of vitamin E on the response to hyperoxia for in vitro lung lipid peroxidation*

	In vitro lipid peroxide formation (nmoles malonyldialdehyde produced per mg protein)	
Age	100% oxygen	100% oxygen + vitamin E
Birth	67.6 ± 18.8[1]	
24 hr	77.9 ± 14.6	57.6 ± 12.1
48 hr	65.8 ± 21.6	48.8 ± 26.0
72 hr	96.0 ± 18.0	22.5 ± 2.5[2]

[1] Mean ± S.E. Experimental conditions and statistical analysis were as described in Table 1.

[2] Statistically significant difference from the 100% oxygen group ($P < 0.01$).

Table 5. *The influence of vitamin E on the response to 100% oxygen of lung homogenate superoxide dismutase activity*

	Superoxide dismutase (ng/mg protein × 10^2)		
Age	Room air	100% oxygen	100% oxygen + vitamin E
Birth	6.40 ± 0.52[1]		
24 hr	6.33 ± 0.61	6.67 ± 0.63	6.35 ± 0.55
48 hr	6.58 ± 0.60	8.55 ± 0.64[2]	6.79 ± 0.42
72 hr	6.47 ± 0.47	9.87 ± 0.64[2]	7.29 ± 0.56

[1] Mean ± S.E. Experimental conditions and statistical analysis were as described in Table 1.

[2] Statistically significant difference from both the room air and 100% oxygen + vitamin E groups ($P < 0.01$). Values from the room air groups did not differ significantly from those of the 100% oxygen plus vitamin E group at any time.

Table 6. *The influence of vitamin E on the response to 100% oxygen of lung cytosol glutathione peroxidase activity*

Age	Glutathione peroxidase (nmoles NADPH/min/mg protein × 10^2) Room air	100% oxygen	100% oxygen + vitamin E
Birth	17.38 ± 1.16[1]		
24 hr	12.75 ± 1.27	22.71 ± 6.20	16.88 ± 4.41
48 hr	16.33 ± 2.80	20.18 ± 2.11	17.45 ± 2.36
72 hr	12.91 ± 1.36	18.16 ± 1.89[2]	14.62 ± 0.98

[1] Mean ± S.E. of five experiments. Experimental conditions and statistical analysis otherwise were as described in Table 1.

[2] Statistically significant difference from both the room air and 100% oxygen + vitamin E groups ($P < 0.05$). Values for the room air group did not differ significantly from those of the 100% oxygen plus vitamin E group at any time.

Table 7. *The influence of vitamin E on the response to 100% oxygen of lung homogenate glutathione reductase activity*

Age	Glutathione reductase (nmoles NADPH/min/mg protein) Room air	100% oxygen	100% oxygen + vitamin E
Birth	13.92 ± 1.56[1]		
24 hr	14.56 ± 2.38	16.10 ± 0.76	14.44 ± 2.19
48 hr	16.65 ± 2.50	18.68 ± 2.99	15.04 ± 3.21
72 hr	14.04 ± 1.25	18.76 ± 2.54[2]	14.23 ± 1.77

[1] Mean ± S.E. Experimental conditions and statistical analysis were as described in Table 1.

[2] Statistically significant difference from both the room air and 100% oxygen plus vitamin E groups ($P < 0.05$). Values for the room air group did not differ significantly from those of the 100% oxygen plus vitamin E group at any time.

controls at any experimental time and were significantly less at 48 and 72 hr than those of the animals exposed to 100% oxygen but not treated with vitamin E.

Findings for lung glutathione peroxidase and glutathione reductase were similar to those for superoxide dismutase and are presented in Tables 6 and 7, respectively. For these glutathione system enzymes, pulmonary activites were consistently highest in the 100% oxygen group at each experimental time. Although these increases did not reach statistical significance at 24 and 48 hr, results for both enzymes at 72 hr were significantly elevated over corresponding results for the two other experimental groups. For animals treated with vitamin E during exposure to 100% oxygen, lung glutathione peroxidase and glutathione reductase activities did not differ from room air controls at any time.

Lung protein content did not vary significantly among the three experimental groups. Injection of the solubilizing vehicle for vitamin E was without effect on either enzyme activity or *in vitro* lipid peroxidation in animals exposed to oxygen.

MORPHOLOGY

The influence on lung morphology of exposure to 100% oxygen for 72 hr is shown in Fig. 1. After 72 hr, lung specimens demonstrated light microscopic evidence of early epithelial injury. This injury was characterized by focal atelectasis peripherally and around bronchioles, with adjacent zones of overexpansion of alveoli and dilation of small airways (emphysema).

When lungs from rabbits receiving vitamin E, 2 mg/100 g/day, were examined after 72 hr exposure to 100% oxygen, there was no consistent morphologic evidence of protection from oxidant injury. However, at a vitamin E dose of 6 mg/100 g/day, lung specimens were indistinguishable after 72 hr oxygen exposure from animals maintained in room air. Figures 2 and 3 are representative photomicrographs from animals from the room air group and the vitamin E treated 100% oxygen group, respectively. As depicted in these photomicrographs, specimens were similar histologically, in that alveoli were of uniform size and shape, alveolar walls were delicate, and no atelectasis or emphysema were observed.

Results of a morphometric quantitation of the percentage of atelectasis and percentage of emphysema are presented in Table 8. Specimens from the 100% oxygen group averaged approximately four times the amount of atelectasis and two times the amount of emphysema seen in the room air and vitamin E-treated groups. In the group exposed to oxygen but treated with vitamin E, the extent of atelectasis and emphysema was not increased significantly compared to room air controls.

DISCUSSION

Prolonged exposure to high concentrations of oxygen will cause damage to pulmonary parenchyma. This oxidant injury appears to be mediated by the generation of free radicals, such as the superoxy radical ($O^{\cdot}_2$), the perhydroxy radical ($HOO^{\cdot}$), and the hydroxy radical ($OH^{\cdot}$), formed *in vivo* during the univalent reduction of molecular oxygen (3, 9, 11, 12). These highly reactive free radicals result in tissue injury through peroxidation of membrane lipids or reaction with sulfhydryl groups to damage enzymes or structural proteins (5, 14, 31).

The ability of organisms to withstand oxidant injury is determined in part by cellular antioxidant defenses. Among the endogenous pulmonary antioxidants are several enzymes which mediate the deactivation of free radicals. Superoxide dismutase catalyzes the reaction $O_2^- + O_2^- + 2H^+ \; H_2O_2 + O_2$ and appears to be a primary cellular defense against the superoxy radical (28). In the glutathione system, glutathione peroxidase inhibits lipid peroxidation by reducing peroxy fatty acids while simultaneously oxidizing glutathione. In a reaction linked to an NADPH-generating system, glutathione reductase then replenishes reduced glutathione (19). The activities of lung superoxide dismutase, glutathione peroxidase, and glutathione reductase have all been shown to increase with hyperoxic stress, and such increases are felt to represent an adaptive response to the greater potential for oxidant injury which accompanies exposure to high concentrations of oxygen (4, 10, 23, 39). The increased activities of superoxide dismutase, glutathione peroxidase, and glutathione reductase found in animals in our 100% oxygen group provide further confirmation that pulmonary antioxidant enzyme activities increase in response to hyperoxia.

Among chemical antioxidants which may protect the lung from oxidant injury is vitamin E. Several investigators have demonstrated that vitamin E deficiency enhances the toxic effects of oxygen upon the lung (18, 27, 32), and Taylor *et al.* (33) has shown that vitamin E treatment of vitamin E-deficient rats significantly decreases lung damage in animals exposed to oxidant stress. Evidence that vitamin E ameliorates bronchopulmonary dysplasia in human infants has also been reported (7).

Although the precise mechanism by which vitamin E functions as an antioxidant has yet to be determined, speculation on its mechanism of antioxidant protection has centered on at least two areas. Lucy (21) has speculated that vitamin E may be a structural component of membranes which makes membrane polyunsaturated fatty acid (PUFA) more resistant to peroxidation. Alternatively, others have provided evidence for a more direct antioxidant role for vitamin E as a tissue free radical scavenger (30).

In our non-vitamin E-treated animals, plasma vitamin E levels rose slightly over 3 days of artificial feedings, reflecting the small amounts of vitamin E which is normally present in corn oil [7 mg tocopherol per 100 g (13)]. Despite this basal dietary vitamin E intake in our untreated animals, the rapid increase in erythrocyte sensitivity to H_2O_2 hemolysis observed in these animals suggests

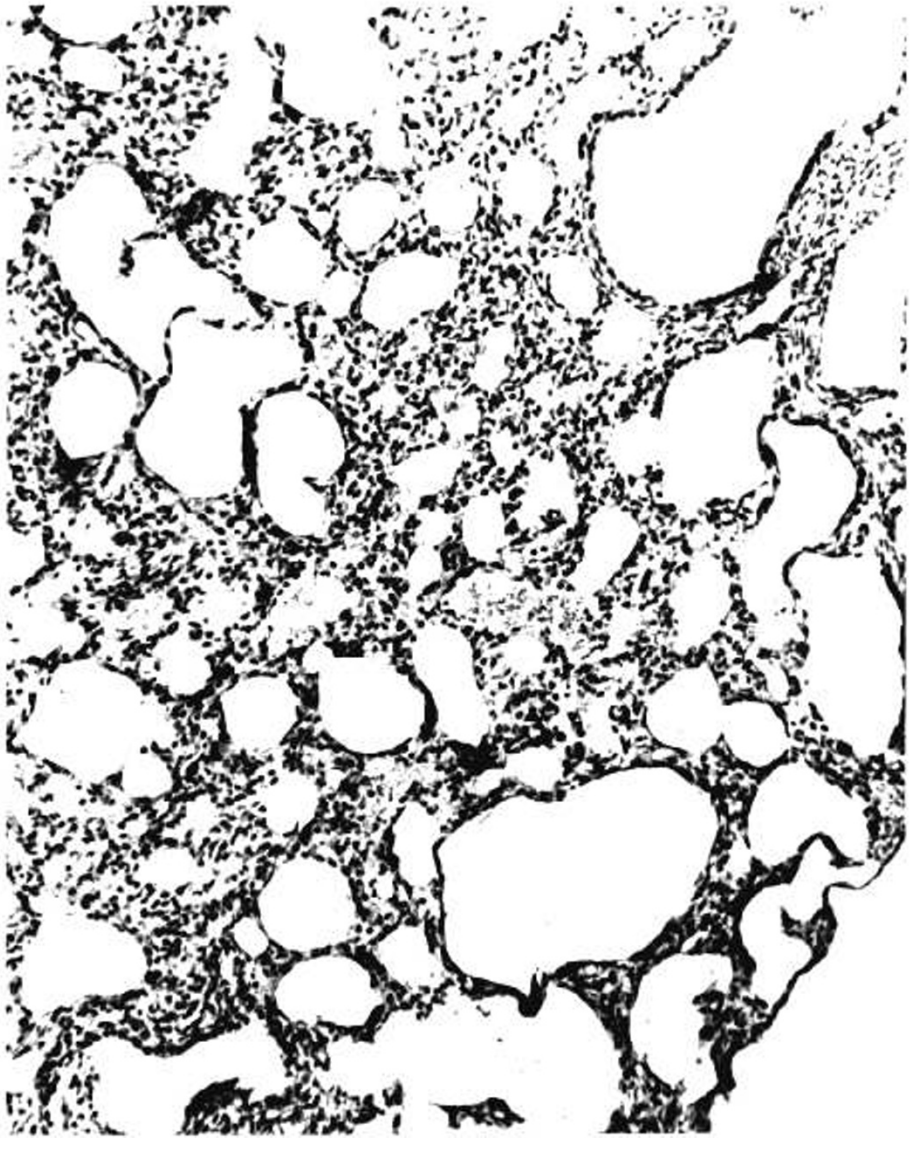

Fig. 1. A light micrograph of lung from a 72-hr-old rabbit maintained in 100% oxygen since birth. Early epithelial injury is apparent and is characterized by focal atelectasis with adjacent zones of alveolar emphysema and small airway dilation. (Hematoxylin and eosin; original magnification, × 140.)

that these artificially fed newborn rabbits quickly manifested chemical evidence of vitamin E deficiency. This deficiency most likely reflects the high PUFA intake from corn oil (56%) linoleic acid (13) in our experimental animals, because high PUFA intake is known to augment requirements for vitamin E (13, 16, 36). Because Frank *et al.* (10) have shown that nursed newborn rabbits are relatively resistant to pulmonary oxygen toxicity and demonstrate only minor morphologic changes after 7 days exposure to 100% oxygen, our morphologic findings of acute epithelial injury after 72 hr of 100% oxygen exposure provide additional evidence that nutritional factors such as vitamin E may alter susceptibility to pulmonary oxygen toxicity (18, 27, 32). The increased susceptibility to oxygen toxicity seen in the present study also may be due to deficiency of factors such as selenium and other antioxidents found in normal milk.

Within this nutritional context, vitamin E was capable of affecting the biochemical and morphologic response to hyperoxia. When our experimental animals were treated with vitamin E (2 mg/100 g/day) while being exposed to 100% oxygen, lung vitamin E content was increased, and the susceptibility of lung tissue to *in vitro* lipid peroxidation was dramatically reduced. These findings suggest that additional lung antioxidant protection was provided by vitamin E treatment.

Additionally, vitamin E treatment diminished the usual hyperoxia-induced increases in the pulmonary antioxidant enzymes superoxide dismutase, glutathione peroxidase, and glutathione reductase. These findings are consistent with the observations of Chow *et al.* (2) who, using ozone as an oxidant stress, have noted that ozone-induced increases in pulmonary antioxidant enzymes are diminished by vitamin E treatment. Both our findings and those of Chow *et al.* suggest that antioxidant protection of lung by vitamin E may involve the inactivation of the likely stimuli for antioxidant enzyme induction, oxygen free radicals. Although a generalized inhibition of cell metabolic activity by vitamin E might be an alternative interpretation of these enzyme findings, we feel this is very unlikely because we have observed that another lung metabolic response to hyperoxia, the hyperoxia-induced increase in the oxidation of glucose to lactate, is unaffected by vitamin E treatment (R. E. Kimura, O. F. Wender, and J. B. Warshaw, unpublished observation).

Although at a dose of 2 mg/100 g/day, vitamin E altered the lung biochemical response to hyperoxia, a protective effect of vitamin E treatment could not be demonstrated at that dose. The discrepancy suggested that at low doses, although vitamin E scavenges sufficient free radicals to blunt the lung biochemical response to hyperoxia, structural damage could continue because

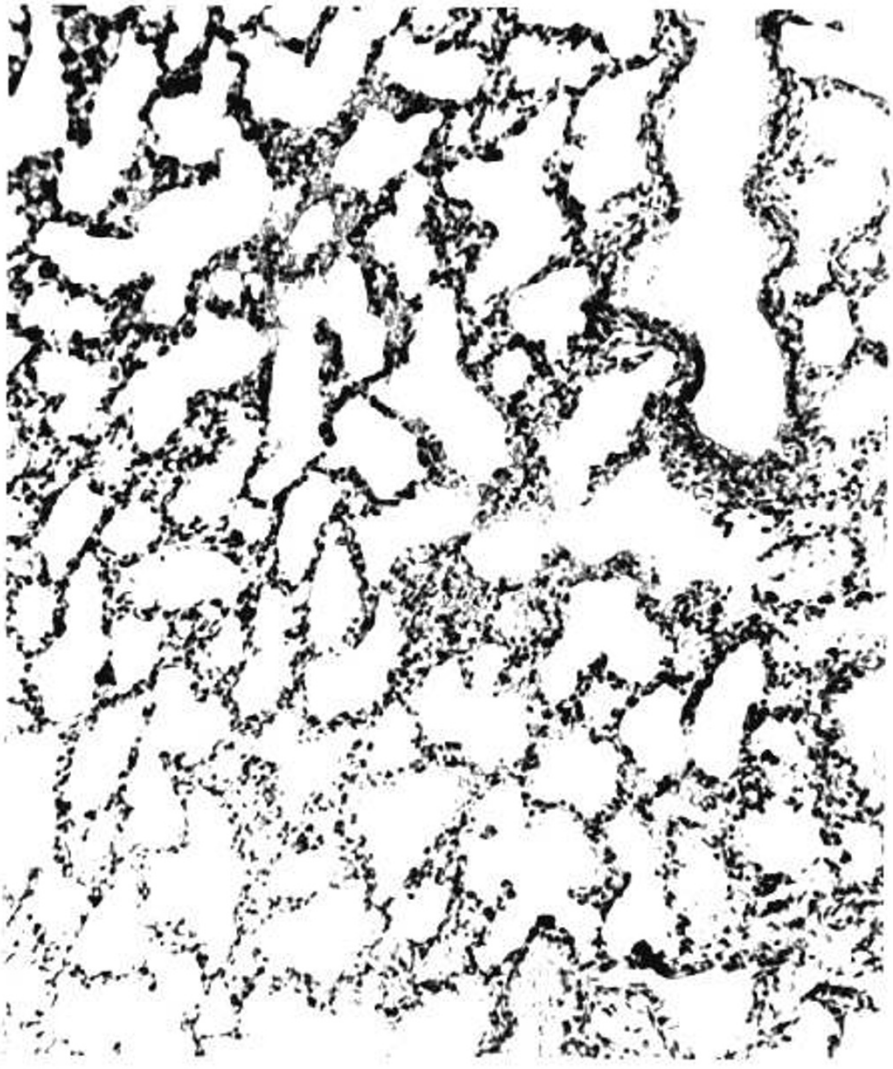

Fig. 2. A light micrograph of lung from a 72-hr-old rabbit maintained in room air since birth. (Hematoxylin and eosin, original magnification × 140).

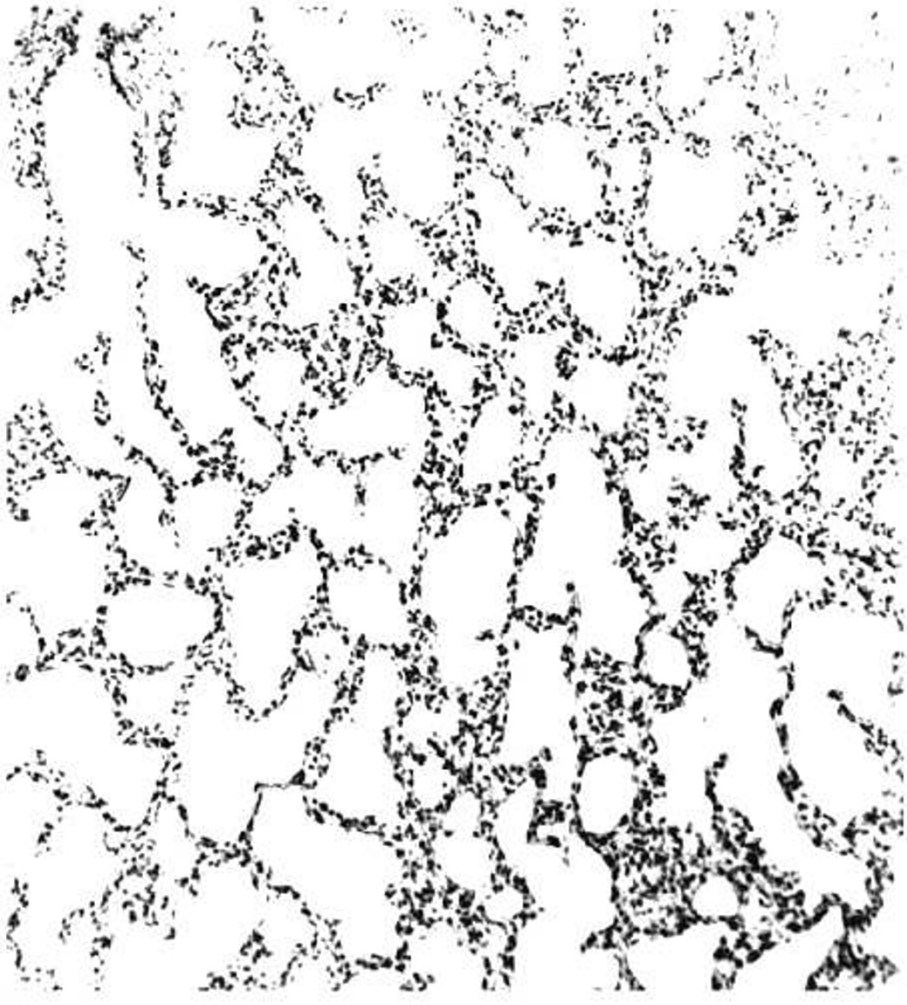

Fig. 3. A light micrograph of lung from a 72-hr-old rabbit treated with vitamin E (6 mg/100 g/day) and maintained in 100% oxygen since birth. This specimen is histologically indistinguishable from Figure 2. Alveoli are of uniform size and shape, alveolar walls are delicate, and no atelectasis or emphysema are observed. (Hematoxylin and eosin, original magnification × 140).

Table 8. *The influence of vitamin E on lung morphometric response to 72 hr exposure to 100% oxygen*

	Morphometric analysis of lung injury (% each histologic specimen)		
	Room air	100% oxygen	100% oxygen + vitamin E
% atelectasis	10.4 ± 1.9[1]	40.3 ± 5.6[2]	9.3 ± 5.5
% emphysema	8.0 ± 2.0	16.4 ± 3.2	7.1 ± 2.5
% normal	81.6 ± 3.5	43.3 ± 8.5[2]	83.6 ± 7.7
Total	100.0	100.0	100.0

[1] Mean ± S.E. Experimental conditions and statistical analysis were as described in Table 1.

[2] Statistically significant difference from both the room air and 100% oxygen + vitamin E groups ($P < 0.01$). The increased percentage emphysema in the 100% oxygen group approached statistical significance ($P = 0.09$).

of free radicals present in excess to available vitamin E. When the vitamin E dose was increased to 6 mg/100 g/day, a protective effect on lung morphology was observed. Taken together with the biochemical findings at a lower vitamin E dose, these morphologic findings of antioxidant protection with vitamin E treatment provide further evidence of an antioxidant effect of vitamin E in lung.

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41. Requests for reprints should be addressed to: Dr. Joseph Warshaw, Department of Pediatrics, Yale University School of Medicine, 333 Cedar Street, New Haven, CT 06510.
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