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Hyperoxic Injury of Immature Guinea Pig Lung Is Mediated via Hydroxyl Radicals

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ABSTRACT

Support of preterm infants with ventilation and oxygen therapy frequently leads to the development of chronic lung disease. Oxidative stress, through the generation of excess oxygen free radicals, is thought to play a major role in this condition. At present the radical species responsible for oxidative lung injury is not known, and effective antioxidant based therapies are not available. The purpose of this study was to determine whether hydroxyl radicals, potent reactive oxygen species, are involved in chronic oxidative lung injury. To obtain this information we developed a animal model of chronic lung injury using the preterm guinea pig and analyzed lung tissue from these pups for *o*-tyrosine, a specific marker of hydroxyl radical attack. In normoxia control pups the pulmonary content of *o*-tyrosine was low during the first 4 wk of life (range 0.11–0.12% tyrosine). Pups maintained in 85% oxygen were found to have increasing lung *o*-tyrosine over this period (d 7, 0.51%; d 14, 0.8%; d 21, 1.28%; d 28, 1.45% tyrosine). From d 21, the nonenzymatic glycosylation end product, *N*-ε-carboxymethyllysine was also present in significantly increased amounts in hyperoxic-exposed pups. These results implicate hydroxyl radicals as a significant oxidizing species in hyperoxic lung injury and provide a basis for understanding collagen deposition in the neonatal lung. (***Pediatr Res* 38: 286–291, 1995**)

Abbreviations

CML, *N*-ε-carboxymethyllysine
FiO_2, fraction of inspired air

The role of oxygen in the development of neonatal lung disease is unequivocal (1). Since the initial proposal by Gerschman *et al.* (2), the evidence implicating oxygen free radicals in hyperoxia-induced lung injury has increased steadily. In 1981 Freeman and Crapo (3) demonstrated that hyperoxic-exposure resulted in increased oxygen radical production in rat lungs. These investigators subsequently showed that augmentation of superoxide dismutase in endothelial cells prevented oxidative injury, implicating superoxide anions in the injury process (4). These findings were examined in newborn infants with lung disease, and superoxide dismutase was found to provide some benefit (5), reinforcing the free radical hypothesis but without providing any further information as to which radicals were involved. Pitkaenen *et al.* (6) added further weight to this hypothesis when they reported increased ethane and pentane levels in expired air of ventilated neonates. Schlenzig *et al.* (7) also reported increased urinary excretion of malondialdehyde in ventilated premature infants which correlated with FiO_2 levels. None of the above clinical studies provided information regarding the oxygen free radical species involved in neonatal lung injury. Indeed, similar studies in animals models with (8) or without (9) free radical scavengers have not helped to characterize the nature of the oxygen species involved.

Conclusive evidence that both superoxide and hydroxyl radicals are involved in oxidative cell injury came from the *in vitro* studies of Zweier *et al.* (10). They demonstrated clearly that high oxygen concentrations led to the increased production of these species in pulmonary endothelial cells. These findings encouraged us to undertake the present study in which we have investigated the involvement of hydroxyl radicals in an *in vivo* model of neonatal lung disease (11). The rationale of the study is based on the principle that hydroxyl radicals attack both free and protein-bound phenylalanine generating *o*-tyrosine (12, 13). To make this study relevant to the human baby with chronic lung disease we first developed a model of chronic oxidative lung injury based on our previous model of acute oxidative lung injury (11). Lung tissue obtained from preterm guinea pigs maintained in normoxic or hyperoxic conditions for up to 28 d was examined for the presence of *o*-tyrosine. In addition, these tissues were also screened for evidence of non enzymatic glycosylation of proteins by determining the presence of CML.

METHODS

Animals. Virgin, female Hartley strain guinea pigs (500 g) obtained from our colony were caged in pairs in a room

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controlled for temperature (22°C) and light 0600–2000 h. Animals had free access to food and water. The estrus cycle of each female was established by daily vaginal smears. Timed pregnancies were established by introducing a male into the cage three days before the next ovulation. The date of ovulation in the successfully fertilized cycle was taken as gestation d 0. By this method, normal gestation ends with birth on d 68, however, previously we have demonstrated that viable preterm pups can be delivered by cesarean section on d 65 of gestation (11). This operation was performed on the mother while she was under halothane anesthesia maintained with oxygen and nitrous oxide (4:1) mixture. After double clamping of the uterine vessels, fetuses were removed individually, rapidly dried, and weighed. All pups were delivered within 5 min of the initial anesthetic administration.

Each litter was divided randomly and pups allocated to purpose built 25-L Perspex incubators receiving 21% oxygen or 85% oxygen at a flow rate of 3.5 L/min. Incubators were continuously monitored for oxygen concentration and temperature (20–22°C) and changed daily for washing and sterilization. Our previous experience with this model of prematurity have been restricted to acute (72–96 h) hyperoxic exposure (11, 14, 15) or acute hyperoxic exposure followed by a recovery phase (9). As we wanted to develop a model of chronic lung disease in the human neonate we developed a protocol centered around maintaining the pups in 85% oxygen for up to 28 d. To minimize fatalities in these experiments all animals were examined visually on a 2-hourly basis between 0700 and 2100 h for the first 14 d. Where necessary, usually between d 3 and 5, some pups were removed to a chamber in which the oxygen concentration was reduced to 60% for a period of approximately 48 h. This judgment was based on the general alertness and mobility of the pups. Those animals which developed severe oxidative lung injury, and who would have died in 85% oxygen, became lethargic and quickly lost body temperature. Reducing their exposure to 60% oxygen for a short period was found to usually ensure their survival and yet not noticeably influence the development of the chronic form of lung injury. Thereafter, until d 28, a time at which animals showed morphologic evidence of pulmonary fibrosis, pups were maintained in 85% oxygen.

A lactating surrogate dam was placed in each cage to feed and foster the pups. These dams, which were very susceptible to oxidative-induced lung injury, were rotated between the 21 and 85% oxygen exposure chambers on a daily basis. This approach prevented fatalities to the mothers and ensured that both normoxia and hyperoxia-exposed pups received the same nutritional source. Pups were nursed by the surrogate dams in this manner for the first 14 d of the 28 d experimental period. Over the first 5–7 d, pups suckled freely but ate little solid food. From d 7 onward the pups began to nibble at the solid chow available in the cage, such that by d 10 they appeared to feed little from the dam. On d 14 the surrogate dams were removed from the cages and the pups were left to fend for themselves over the next 2 wk.

Collection of lung samples. At 7, 14, 21, and 28 d groups of pups (n = 4/5) were anaesthetized by intraperitoneal injection of pentobarbitone (50 mg/kg). After onset of adequate anesthesia, the abdominal cavity was opened and a blood sample was collected by cardiac puncture. The lungs were then removed *en bloc*, rinsed in sterile saline, and stored at 70°C until analysis.

Determination of o-tyrosine. Samples were weighed and hydrolyzed using 6 N HCL at 110°C for 16 h. The hydrolysate was spun down at 5000 × g at room temperature. Aliquots (50 μL) of the hydrolysate were run by reverse phase HPLC as described by Ishimitsu *et al.* (16) for serum samples. With this approach no interfering peaks to the *o*-tyrosine where found to be present in lung hydrolysates. The column used was a Supersphere, 4 μm, 100 × 4.6 mm. The HPLC pump was a Shimadzu LC6A, the sampler was a SCL-68 (Shimadzu), the integrator a CR4AX, and the detector a Jasco Flurometer 820 FP. The excitation was at 275 nm; the emission was at 305 nm. Coefficient of variation for *o*-tyrosine standards was 2–3%, whereas that for samples was 8%.

Determination of CML. The determination of CML was performed using aliquots of hydrolysates used for *o*-tyrosine studies. The method given in a previous publication (17) was used, briefly: the evaporated hydrolysate was redissolved for ion exchange chromatography on Dowex W 50 as given in a previous publication (18). The HPLC determination of CML was performed by HPLC after precolumn derivatization using *o*-phthalaldehyde and 3-mercaptopropionic acid. The column was a Hypersil ODS, 2 μM, 25 × 4 mm. The mobile phase was eluent A: 25 mM MaOAc, pH 7.2, with 0.7% THF and B: 100 mM NaOAc, pH 7.2, in acetonitrile (1.4 vol/vol). The gradient applied was 0–30% B, from 0 to 9 min; 30–50% B, from 9–11 min; 50–100% B, from 13–14 min; 100% B, from 14–18 min; 100–0% B, from 18–19 min. The flow rate was 0.8 mL/min. Detection was at 230 nm excitation and 450 nm emission. Coefficient of variation for standards was 5.6% and for samples 9.4%.

Light and electron microscopy. After 28 d of exposure to either 21 or 85% oxygen, pups (n = 3/group) were anesthetized by intraperitoneal injection of pentobarbitone (50 mg/kg). After the onset of deep anesthesia, the trachea was isolated, and a 14-gauge cannula inserted and secured. The thoracic cavity was then opened and the lungs inflated with air to a constant pressure of 10 cm H_2O. The lungs were then perfused via the right ventricle for 60 min with half-strength Karnovskys fixative. The heart and lung preparation was then dissected from the thorax *en bloc* and immersed in full-strength Karnovskys fixative (2.5% glutaraldehyde and 2.5% paraformaldehyde) for 24 h. The left caudal lobe was then bisected from the hillum to the periphery, and the facing sections were processed for light and electron microscopy.

For light microscopy, 4-μm sections were cut and stained with hematoxylin and eosin and Martius scarlet blue. For electron microscopy, four tissue pieces were cut from the central portion of the caudal lobe, stored overnight in cacodylate/sucrose buffer (pH 7.4, 0.85 osmol/L), postfixed in 2% (wt/vol) osmium tetroxide for 2 h, and then stained *en bloc* with 1.5% (wt/vol) uranyl acetate before being embedded in Spurr resin. Two of these blocks were then selected at random for thick (0.5 μm) sections, which were stained with toluene blue and an appropriate area was selected for thin sectioning

Table 1. *The effect of oxidative stress on o-tyrosine content of immature guinea pig lung*

Treatment	Time (d)	Lung weight (g)	*o*-Tyrosine (%)	Tyrosine (μmol/g wet wt)	Phenylalanine (μmol/g wet wt)
21% Oxygen	7	1.8 ± 0.3	0.12 ± 0.01	7.3 ± 0.6	17.3 ± 2.8
	14	1.8 ± 0.3	0.12 ± 0.01	7.3 ± 0.5	18.9 ± 2.6
	21	2.1 ± 0.4	0.11 ± 0.01	7.1 ± 0.5	18.1 ± 1.9
	28	2.6 ± 0.3*	0.11 ± 0.01	8.1 ± 0.8	19.4 ± 3.3
85% Oxygen	7	2.7 ± 0.5*	0.51 ± 0.11*†	7.8 ± 0.1	18.2 ± 3.0
	14	4.0 ± 1.0*†	0.84 ± 0.15*†	8.4 ± 1.4	19.2 ± 2.9
	21	4.3 ± 0.5*†‡	1.28 ± 0.23*†‡	8.2 ± 0.5	17.9 ± 3.2
	28	4.6 ± 0.9*†‡	1.45 ± 0.10*†‡	8.7 ± 1.8	19.9 ± 3.1

Superscripts indicate statistically significant differences ($p < 0.05$ or better) as follows:
* Different from d 7 or d 14, 21% oxygen.
† Different from d 21 or d 28, 21% oxygen.
‡ Different from d 7, 85% oxygen.

(0–90 nm). Thin sections were mounted on high transmission copper grids and were stained with lead acetate. Each thin section was qualitatively assessed and photographed on an Hitachi 7000 electron microscope (Hitachi, Tokyo, Japan).

Statistical evaluation of the results. Differences between oxygen exposed and control animals were analyzed by analysis of variance using general linear model analysis (SAS User's Guide: Statistics, version 5 ed. SAS Institute Inc, Cary, 1985).

RESULTS

Survival. All pups delivered at d 65 of gestation by caesarean section developed respiratory distress, characterized by tachypnoea and rib retraction. This initial distress generally resolved within 12 h. Those pups maintained in 85% oxygen also experienced a second period of respiratory difficulty, usually between 3 and 5 d post delivery. A series of pilot studies revealed that if the oxygen concentration in the chamber was reduced to 60% for up to 48 h then the majority of pups would survive this second period of distress. Animals that survived this period of respiratory difficulty had no further life-threatening episodes. Eighty five percent of those pups maintained in 21% oxygen and 68% of those kept in 85% oxygen survived the 28-d experimental protocol.

Body weight and lung weight. After an initial fall in body weight after birth, pups in both treatment groups gained weight over the 28-d experimental period. Those pups maintained in 85% oxygen, however, grew at a reduced rate. A significant difference in body weight between the two groups was first noted at d 7, such that by d 28, pups maintained in 85% oxygen were 20% lighter than the air-exposed controls. Lung weight increased with age, in 21% oxygen-exposed pups it increased 45% over the 28-d period. Lung weight increased significantly in pups exposed to 85% oxygen compared with control pups from d 7 onward (Table 1).

Lung histology. After 28 d in 21% oxygen the lungs of preterm guinea pigs were structurally normal with the alveoli separated by thin septa, and there was no evidence of injury (Fig. 1, *A* and *B*). After exposure to 85% oxygen there were a number of structural alterations to the pulmonary architecture. Comparison of Figure 1, *A* and *C*, demonstrates a thickening of the interstitium in hyperoxic exposed animals. This change is seen more closely at higher magnification (compare Fig. 1, *B* and *D*) and is due to both hypertrophied cells and deposits of protein. Examination of lung sections by electron microscopy revealed large numbers of fibroblasts and extensive collagen deposits in the septa of pups maintained in 85% for 28 d (Fig. 2, *B*, *C*, and *D*).

o-Tyrosine. The tyrosine concentration in lung remained constant in both normoxic and hyperoxic exposed pups over the 28-d experimental period. As lung weight increased significantly in pups maintained in 85% oxygen it follows that the tyrosine content of these lungs also increased in comparison to that in the lung of pups maintained in 21% oxygen. A small proportion of tyrosine (0.12%) was present in the form of *o*-tyrosine in normoxic exposed pups (Table 1). Lung *o*-tyrosine content did not change in normoxic exposed-pups over the 28-d period. The proportion of tyrosine present as *o*-tyrosine increased significantly from d 7 onward in those pups maintained in 85% oxygen (Table 1).

CML. Glycoxidation, expressed as the concentration of CML in the lung, did not change over the first 28 d in control pups. In pups maintained in 85% oxygen for 7 d, CML concentration was similar to that in control animals (Fig. 3). However, by d 21, lung CML content had increased significantly in hyperoxic, exposed pups, and it increased further in pups maintained in 85% oxygen for 28 d (Fig. 3).

DISCUSSION

In this study, an animal model of chronic lung disease was developed and used to determine whether hydroxyl radicals were involved in oxidative lung injury. The animal model used, the preterm guinea pig, has been previously reported for studies of acute oxidative lung injury (11). In the present study, a protocol was devised using chronic exposure to 85% oxygen. Although careful monitoring of the animals is required over the first 7 d with this protocol, those which survive beyond this period are usually robust enough to complete the 28-d protocol.

Examination of lung tissue from pups exposed to 85% oxygen for 4 wk, by light and electron microscopy, revealed marked changes in pulmonary architecture that are characteristic of many of the features observed in infants suffering from chronic lung disease. These included interstitial edema, pulmonary fibrosis and inflammation, squamous metaplasia and hyperplasia of airways (data not shown), and injury to the

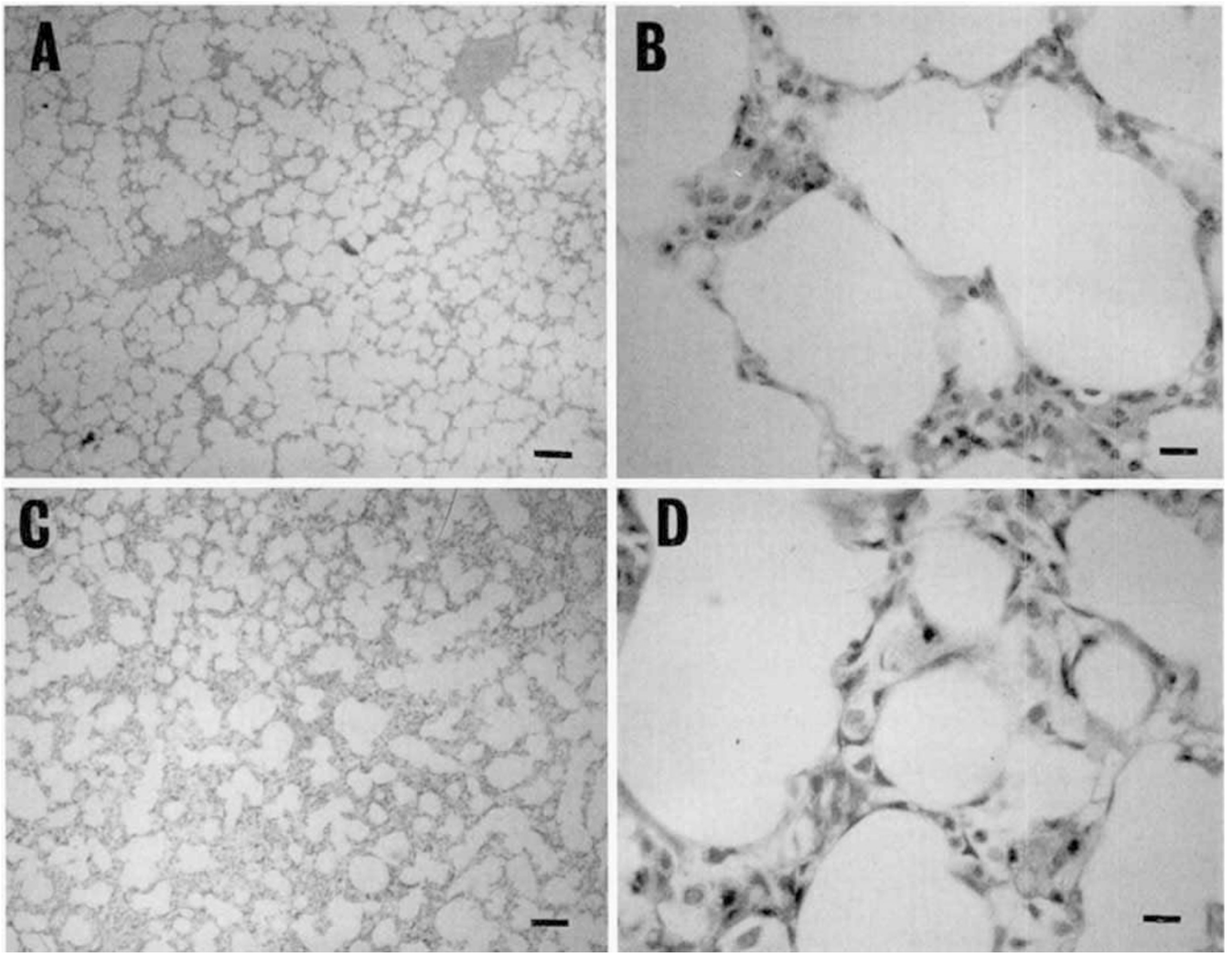

Figure 1. Sections of lungs from preterm guinea pugs exposed to either 21% oxygen (*A* and *B*) or 85% oxygen (*C* and *D*) for 28 d stained with Martius scarlet blue. Bars represent 50 μm for *A* and *C* and 25 μm for *B* and *D*. Sections *A* and *B* show normal thin septa between alveoli. Sections *C* and *D* demonstrate thickened septa as a consequence of both fluid accumulation and protein deposition.

pulmonary endothelium. As chronic lung disease of the preterm neonate involves increased accumulation of connective tissue proteins, the marked accumulation of connective tissue in lungs of hyperoxic-exposed pups was of specific interest in present study.

It is likely that these connective tissue changes arise as a result of a number of responses of the immature lung to oxidative injury (1). One of the initial steps involved is increased free radical production. As a result, pulmonary proteins become oxidized, both directly and indirectly during hyperoxic exposure. The finding that *o*-tyrosine, a specific marker of hydroxyl radical attack (12, 13), was present in low concentrations in pups maintained in normoxia, and in high concentrations in lung hydrolysates of preterm guinea pups treated with 85% oxygen, supports the concept that hydroxyl radicals play an important role in the injury process.

Interestingly, *o*-tyrosine residues accumulated in a time-dependent fashion. At d 7 there was a 4-fold increase in *o*-tyrosine content of lung, whereas at d 28 this had increased to a 13-fold difference. We interpret these data as indicating that hydroxyl radical-mediated oxidation of phenylalanine residues in protein occurred at a faster rate than the clearance of *o*-tyrosine. Such an interpretation is supported by data which indicates that, in the immature lung, degradation rates are considerably less than synthetic rates, a situation that permits rapid growth and development of the immature lung (19). In addition, the finding that, *in vivo*, protein synthetic pathways are extremely sensitive to oxidative stress (20, 21) does not exclude the possibility that degradation pathways may also be sensitive to oxidative stress.

Although the findings of the present study strongly suggest hydroxyl radical formation during hyperoxic lung injury, the possibility remains that additional, as yet unrecognized, radical species may also be involved in the injury process. These present findings regarding hydroxyl radical formation *in vivo* are, however, supported by similar findings *in vitro* by Zweler *et al.* (10) using electron paramagnetic resonance. Using the spin trap 5,5′-diemethyl-L-pyrroline-*n*-oxide to detect free oxygen radical generation in hyperoxic pulmonary endothelial cells, they demonstrated that the hydroxyl radical scavenger, ethanol ethyl, decreased the spin trap signal. Moreover, they found that hydroxyl radical generation was derived from su-

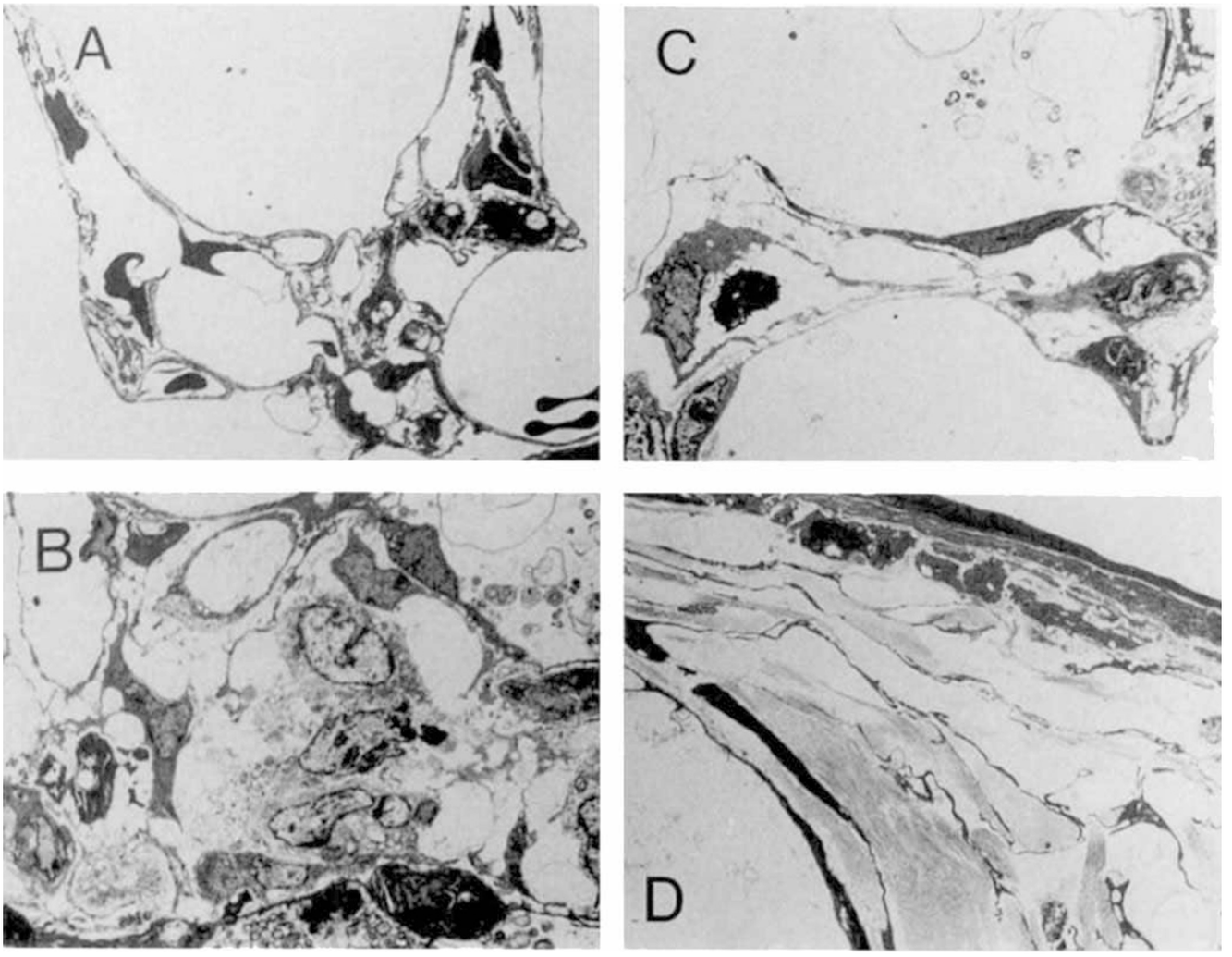

Figure 2. Electron micrographs of lungs from preterm guinea pigs exposed to either 21% oxygen (*A*) or 85% oxygen (*B*, *C*, and *D*). Section *A* shows thin septa in air-exposed pups. Increased interstitial thickening as a consequence of fluid and cell accumulation are seen in hyperoxia-exposed pups (sections *B*, *C*, and *D*). A neutrophil is present in the capillary in section *C*. Collagen deposits are also evident (magnification ×6000 *A–C*; ×8000 *D*).

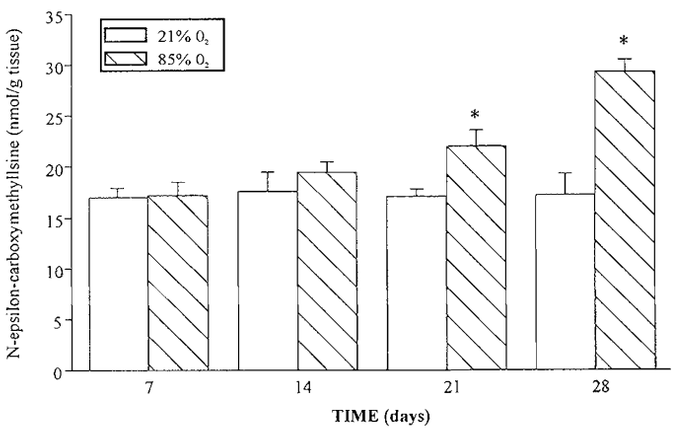

Figure 3. Concentration of CML in lungs of pups maintained in either 21% oxygen (normoxia) or 85% oxygen (hyperoxia) for up to 28 d. $^*p < 0.05$.

peroxide radicals as the signal was also quenched by superoxide dismutase (10). This finding supports previous studies which reported beneficial effects of superoxide dismutase *in vivo* (22).

Although impressive evidence exists verifying the powerful antioxidant properties of vitamin E *in vitro*, the relevance of these findings *in vivo* remains a matter of conjecture. After nearly 30 y of intensive research employing a variety of animal models (23, 24) coupled with numerous clinical trials in infants (25), there is little evidence to support a benefit of vitamin E supplementation in individuals who are already vitamin E replete. These present data throw some light on why supplementation with this antioxidant alone is ineffective in the prevention of, and treatment of, chronic lung disease in babies. Vitamin E will not prevent the formation of highly reactive hydroxyl radicals. Likewise when formed, these species attack numerous cell components causing widespread tissue injury and subsequent connective tissue deposition that would not be prevented by vitamin E alone.

Hydroxyl radicals attack many cell components including DNA, causing strand breaks and oxidation, both of which have serious consequences (26). As shown above, proteins are also modified and inactivated. This is mainly by the oxidative cleavage of tryptophan, histidine, and phenylalanine residues, but glycoxidation of lysine also occurs (27). In the present

study, gylcoxidation of protein was demonstrated as increased CML content of lungs in hyperoxia-exposed pups from 21 d onward. This observation is supported by previous finding of Baynes (28) who demonstrated similar complications in diabetes and aging, two conditions that are also recognized to be associated with oxidative stress.

In conclusion, the results obtained with the aromatic hydroxylation of phenylalanine have revealed increased hydroxyl radical formation in hyperoxic lung injury. Although these data do not exclude the involvement of other radical species, they do strongly implicate hydroxyl radical in the pathogenesis of hyperoxic lung injury. Potential therapeutic approaches to treat this condition may therefore include superoxide dismutase, combined with metal chelators and sulfur-containing amino acids which react rapidly with hydroxyl radicals (29). Using the animal model of chronic lung disease developed in this study we are now in the process of testing this hypothesis.

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