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Comparative Responses of Premature *Versus* Full-Term Newborn Rats to Prolonged Hyperoxia

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ABSTRACT. Because fetal rat lungs have lower baseline levels of both surfactant and antioxidant enzymes than full-term newborn rats, we questioned whether prematurely delivered rats might be more susceptible to O_2 toxicity than those born at term. In the present studies, prematurely delivered rats (gestational d 21 of 22) and full-term rat pups were simultaneously put in >95% O_2 after birth. Surprisingly, we found that the preterm rats were not more susceptible to O_2-induced lung damage and lethality than full-term newborns, but, in fact, the composite percentage of survival was even greater in the preterm pups from 7 to 9 d in hyperoxia and were similar thereafter up to 14 d in high O_2. In addition, the preterm rats showed significantly decreased lung wet/dry weight ratios and consistently less severe pathologic evidence of pulmonary edema compared with term rats at 6 and 8 d of O_2 exposure. The premature pups demonstrated the capability of inducing pulmonary antioxidant enzyme responses to hyperoxia by 3 d, and had significantly elevated copper-zinc superoxide dismutase, catalase, and glutathione peroxidase activities (and lung surfactant contents) at 6 d of O_2 exposure compared with the term rats in O_2. The rates of lung total O_2 consumption and cyanide-resistant O_2 consumption at d 6 in hyperoxia were not different for preterm *versus* term pups. Although the basis for the transiently improved survival and decreased evidence of pulmonary O_2 toxicity in the preterm rats is presently unknown, these findings clearly indicate that premature animals of at least one species are equally able to induce protective lung antioxidant enzymes and surfactant responses to hyperoxia as full-term newborn animals. (*Pediatr Res* 35: 233–237, 1994)

Abbreviations

AOE, antioxidant enzyme
DSPC, disaturated phosphatidylcholine
TPL, total phospholipid

Neonatal animals of many species are relatively resistant to pulmonary oxygen toxicity and lethality when compared with adult animals of the same species (1). This relative O_2 tolerance is manifested by much longer survival in hyperoxia and is associated with the induction of increased lung AOE (superoxide dismutase, catalase, and glutathione peroxidase) activities during O_2 exposure (1). Augmented AOE activity levels have been consistently associated with protection from hyperoxic exposure, and conversely, the failure to increase protective AOE activities during high O_2 challenge usually results in susceptibility to severe O_2-induced lung damage (and lethality) (2–4).

Because fetal rats have lower baseline levels of both surfactant and pulmonary AOE compared with full-term newborn rats (5), we questioned whether premature rats might be more susceptible to O_2 toxicity than those born at term. To answer this experimental question, which to date has been previously explored in only two other species (rabbit and guinea pig), we performed studies in which term-newborn and prematurely delivered rats were simultaneously exposed to prolonged hyperoxia. Comparative AOE and surfactant responses to O_2 challenge were determined, as were comparative assessments of O_2-induced lung damage. The findings herein indicate an ability of the preterm rat to increase pulmonary AOE and surfactant in response to hyperoxia, with a transiently improved survival rate and decreased evidence of pulmonary O_2 toxicity compared with the full-term newborn of this species.

MATERIALS AND METHODS

Animals. We have had an ongoing rat-breeding program in our laboratories for the last 10 y. Adult female and male Sprague-Dawley albino rats are obtained from Charles River Laboratories (Wellington, MA). Breeding is accomplished by placing two female rats in the same cage with one male overnight. Probable pregnancy is determined by a positive vaginal sperm smear the next morning, and the onset of gestation is considered to be the midpoint of the cohabitation period. The timed-pregnancy rats are maintained on standard laboratory food and water *ad libitum* and are kept on a 12-h light/dark cycle. Pregnant rats were either allowed to deliver normally (d 22 of gestation) or underwent cesarean section on gestational d 21. For our premature group, we selected d 21 of the 22-d gestation period because at this gestational age the animals are premature enough to satisfy the definition of prematurity yet mature enough so that the majority of them are able to survive without the need for vigorous supportive care (mechanical ventilation). Pregnant rats on d 21 of gestation were anesthetized with ketamine/xylazine anesthesia (ketamine/xylazine, 90:10 mg/kg), a rapid hysterotomy was performed, and the newly delivered 21-d gestation rats were rapidly resuscitated by removing the surrounding membranes, drying them vigorously, warming them on heating pads, and applying low-flow O_2 for ~30 min. Initial survival at 30 min averaged 70%.

Hyperoxic exposure. Shortly after delivery, all the live 21-d premature rats from three to five litters were pooled together before being randomly redistributed to surrogate newly delivered (full-term) mother rats. One half of the litters were then placed in >95% O_2 exposure chambers and one half remained in room air. Simultaneously, newly delivered full-term rat pups (obtained within 12 h after birth) were divided in the same way into hyperoxic and room air groups. Some nonexperimental newborn rat pups were added to the preterm groups at 24 h and 48 h of

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exposure to readjust the litter sizes to 10–12 pups per dam to ensure having equal litter sizes for the preterm and full-term groups from the beginning of the exposures (for equivalent nutrition).

Exposures to hyperoxia were conducted in 3.5-cubic-foot, clear plastic exposure chambers with continuous monitoring of O_2 concentration (96 to 98% O_2; oxygen analyzer, model OM-11, Beckmann Instruments, Inc., Fullerton, CA). The temperature in the chambers was 23–26°C (in-chamber thermometers), and the humidity was 50–70% (in-chamber hygrometers). The chambers were opened daily (10–15 min) for animal maintenance purposes, weighing the rat litters, and switching the dams from O_2 litters to air litters to prevent the development of O_2 toxicity in the nursing dams. All of the animal experimental protocols were preapproved by the university's Committee on Research Animal Welfare.

Biochemical analyses. For lung AOE analysis, some of the experimental pups from each group were killed by an intraperitoneal overdose of pentobarbital after 3 or 6 d of either hyperoxia or room air exposure. After the great vessels in the abdomen were severed to exsanguinate the animals and the left atrial appendage snipped off to facilitate drainage, their lungs were rapidly perfused *in situ* with ice-cold saline via the pulmonary artery until white. The perfused lungs were then excised, stripped of nonpulmonary tissue, and two to three right lungs or two to three left lungs were pooled per sample and quickly frozen in liquid nitrogen before storage at −70°C for later analyses. For AOE activity assays right lung samples from each group were homogenized in cold 2.5 mM potassium phosphate buffer (pH 7.8) in an Omni homogenizer (Omni International Inc., Waterbury, CT). The homogenates were centrifuged at 27 000 × *g* for 45 min at 4°C. The supernatant fractions were then dialyzed overnight against three changes of 50 vol of 2.5 mM potassium phosphate buffer (pH 7.8) with 0.1 mM EDTA and were subsequently used for analysis of AOE activities using standard spectrophotometric assays for superoxide dismutase (cytochrome *c* reduction rate by xanthine–xanthine oxidase) (6), catalase (H_2O_2 consumption rate) (7), and glutathione peroxidase (NADPH oxidation rate) (8). The method of diethyldithiocarbamate treatment was used to quantitate cytosolic copper-zinc superoxide dismutase and mitochondrial manganese superoxide dismutase activities (9), according to the protocol previously described in detail (10).

For DSPC and TPL determination, aliquots of left lung homogenates (homogenized in cold saline) were subjected to lipid extraction using the method of Bligh and Dyer (11). The extracts were dried under nitrogen, reconstituted with chloroform/methanol (2:1), and assayed for TPL using the method of Morrison (12). A portion of the dried lipid extracts were used to isolate DSPC using the method of Mason *et al.* (13) and then assayed for inorganic phosphorus as described above (12). A known quantity of ^{14}C-dipalmitoyl-phosphatidylcholine (New England Nuclear, Boston, MA) was added before lipid extraction to estimate and correct for sequential losses. Lipids were expressed as mg/g of wet lung weight.

In a single experiment, we measured oxygen radical production using the method of Freeman *et al.* (14), which is based on determining the rate of cyanide-resistant O_2 consumption. Rat lungs were perfused with 10 mM potassium phosphate buffer (pH 7.4) with 150 mM KCl and homogenized in 50 mM potassium phosphate buffer. The samples were then centrifuged at 600 × *g* for 10 min, and the supernatant was used to measure O_2 consumption. To 1.5 mL sample was added 1.5 mL 50 mM potassium phosphate buffer and 30 μL 100 mM NADH; the mixture was then equilibrated with air at 30°C, and the rate of total O_2 uptake was measured with a YSI model 5300 oxygen monitor equipped with standard stirring chambers and Clark O_2 electrodes (Yellow Springs Instrument Company, Inc., Yellow Springs, OH) and a flatbed recorder. The electrode was then removed, 30 μL 100 mM sodium cyanide added, the mixture reequilibrated with air, and the rate of cyanide-resistant oxygen uptake measured. Total and cyanide-resistant O_2 consumption were expressed as nmol O_2/min/mg DNA. DNA in aliquots of the lung homogenates used for AOE activity or for O_2 consumption was extracted and measured spectrophotometrically with purified calf thymus DNA (Sigma Chemical Co., St. Louis, MO) as a standard (15).

Lung pathologic analysis. Randomly selected animals from each group were killed as above and then had their unperfused, uninflated left lung lobes tied off and removed for assessing lung wet weight/dry weight ratios [80°C oven; lung dried until constant weight found (72 h)]. For microscopic studies, their right lungs were then fixed *in situ* via a tracheal cannula at a constant inflation pressure of 20 cm H_2O (fixative, 10% buffered formalin). The coded hematoxylin and eosin-stained lung sections were examined by two investigators for pathologic changes, looking for evidence of interstitial, perivascular/peribronchiolar, and intraalveolar edema.

Statistical analyses. Survival data and incidence of intraalveolar edema were compared by χ^2 testing. For quantitative comparisons of parameters in preterm *versus* term rat pups, *t* test was conducted. Four group comparisons (premature rats air + O_2, newborns air + O_2) were made by analysis of variance and Duncan's multiple range test. Kramer's extension of Duncan's test was used in cases of unequal number of replications (16). For all statistical tests, a $p < 0.05$ value was considered significant difference between the compared values.

RESULTS

The immaturity of the rat pups delivered 1 d before term and used for our comparative hyperoxic studies was confirmed by the data in Table 1. The 1-d preterm rats demonstrated statistically significant decreases in their body weight (↓20%) and lung-tissue DSPC and TPL contents (↓25%) *versus* those values for the full-term rats. The initial mortality rate shortly after premature delivery (30%) and the subsequent mortality rate of the premature pups in room air within 48 h (50%) also indicated the immaturity of these preterm rats compared with the ~100% room air survival of full-term rat pups.

Survival in hyperoxia. In each of six separate hyperoxia exposure experiments, all full-term rat pups were alive in hyperoxia at 48 h. For simultaneously O_2 exposed preterm rats, the survival rate was 90% at 24 h and 82% at 48 h (Fig. 1*A*) ($p < 0.001$ compared with the 50% survival rate at 48 h in the preterm rat pups maintained in room air). Because the preterm air controls had a much higher mortality rate within 48 h, the early deaths in the O_2 preterm group were not considered to represent O_2 toxicity–related deaths. For this reason and because the survival of the preterm pups was very stable from 48 h of O_2 exposure and no further deaths occurred after 48 h in the preterm air controls, we started counting the 14-d survival rates in hyperoxia beginning at 100% for both O_2 groups at d 2 for the composite survival percentages that are illustrated in Figure 1*B*.

As Figure 1 shows, the preterm rats were *not* more susceptible

Table 1. *Comparative parameters of maturation in 1-d preterm vs full-term rat pups**

	Preterm	Term
Body weight (g/pup)	5.35 ± 0.06 (27)†	6.71 ± 0.07 (20)
Lung DSPC (mg/g lung)	3.67 ± 0.16 (9)†	5.11 ± 0.12 (9)
Lung TPL (mg/g lung)	17.33 ± 0.50 (9)†	23.50 ± 0.61 (9)
Survival in air		
24 h	107/137 (78%)†	115/116 (99%)
48 h	69/137 (50%)†	114/116 (98%)

* Values are means ± SEM for (*n*) samples/group. For body weight values, (*n*) = number of litters evaluated. Survival rates do not include the initial mortality (30%) just after birth for preterm group.

† $p < 0.01$ or less for preterm *vs* term.

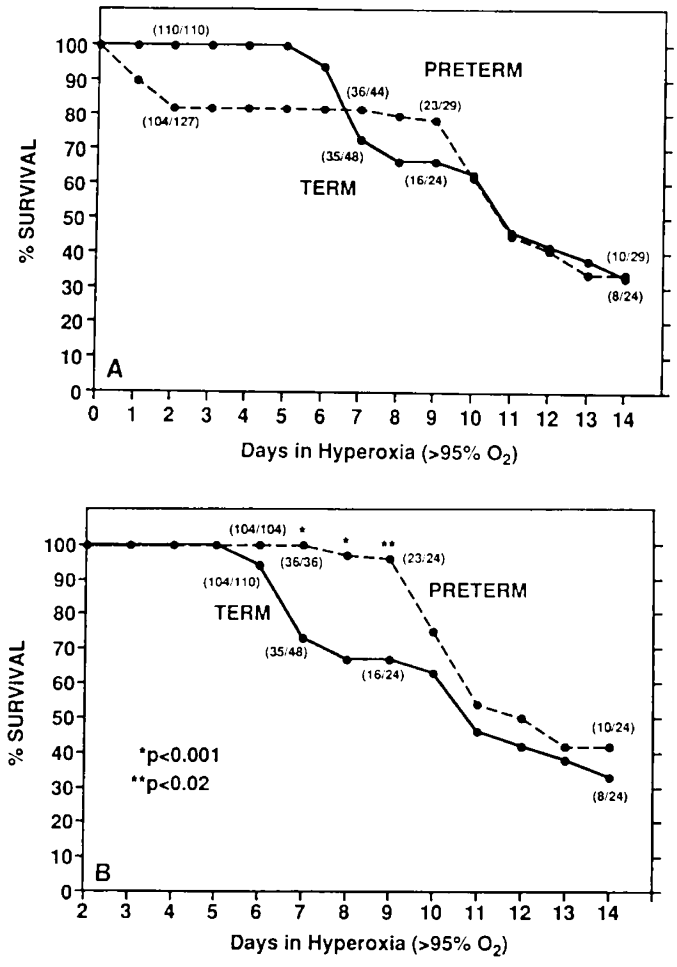

Fig. 1. *A*, Survival of prematurely born and full-term rat pups in >95% O_2 for 14 d (*n/n*, number alive/number put in O_2). For room air controls, survival rates for preterm rats were 78% (at 24 h), 50% (at 48 h), and 50% (2 to 14 d of exposure). For air control term rats, survival was ~100% (0 to 14 d of exposure). Note that the initial decrease in survival of the O_2 preterm rat group at 24 and 48 h is *not* considered to be caused by hyperoxic-induced lethality. *B*, Survival of prematurely born and full-term rat pups in >95% O_2 for 14 d. Because the early deaths in the premature rat pups were not considered to represent O_2 toxicity-related deaths, we started counting survival in this figure as 100% in hyperoxia for both rat groups on d 2. The preterm pup survival rates are significantly improved compared with full-term pups at the time periods from 7 to 9 d in hyperoxia ($p < 0.02$ or less).

to O_2-induced lethality than the full-term newborns but actually had transiently improved O_2 survival rates *versus* full-term pups between exposure d 7 and 9, and the comparative survival rates were then essentially similar during the rest of the period in hyperoxia.

In addition to improved survival *per se*, the preterm rats had other evidence of superior O_2 tolerance around the 1-wk time period in hyperoxia by comparative lung wet weight/dry weight ratios (reflection of pulmonary edema) and comparative light microscopic pathologic findings (Fig. 2). At 8 d of hyperoxia, the lung wet weight/dry weight ratio averaged only 1% higher than the air control values in the preterm pups *versus* 9% higher than the air control values in the term rats ($p < 0.01$). As more definitive of advanced O_2 toxicity, the light-microscopic examination showed a significantly greater incidence of intraalveolar edema in the term pup lungs (34% of lung sections examined) compared with the preterm pups (10%) after 8 d, as well as after 6 d of hyperoxia (17% *versus* 2%). By 14 d in hyperoxia, neither lung wet weight/dry weight nor light microscopic pathologic analysis revealed any significant differences between the preterm rats and term pups, both groups having evidence of severe lung edema (Fig. 2).

Lung biochemical responses to hyperoxia. The comparative responses of the pulmonary AOE to hyperoxic exposure in the two groups of O_2-challenged offspring are shown in Figure 3 (at 3 d of exposure), and the comparative AOE and lung surfactant responses (at 6 d of exposure) are given in Table 2. After 3 d in hyperoxia, the premature rats showed significantly elevated pulmonary copper-zinc superoxide dismutase, catalase, and glutathione peroxidase activities compared with air control pups (Fig. 3). At 6 d of high O_2 exposure, these AOE activity responses were found to be further enhanced in both preterm pups and term rats. In addition, both O_2 groups were able to manifest significantly elevated lung DSPC increase in >95% O_2 (Table 2).

The rates of lung total and cyanide-resistant O_2 consumption of preterm and term animals after 6 d of hyperoxic exposure were not different between the two groups (8.4 ± 0.3 and 4.3 ± 0.3 nmol O_2/min/mg DNA, respectively, for preterm pup lungs *versus* 8.8 ± 0.6 and 4.3 ± 0.2 for term pup lungs; mean ± SEM, $n = 4$ samples/group, $p > 0.05$).

DISCUSSION

Many studies have been reported recently about the developmental maturation of the AOE defense system in the late-gestation fetal lung. It has been demonstrated in each of the five different species examined to date—the rat, rabbit, guinea pig, hamster, and sheep—that the maturation of the lung AOE system and the surfactant system share a chronologically similar gestational pattern of development. Normally, the developing lung markedly increases both its surfactant content and its AOE activity levels during the final 10 to 15% of gestation (5, 17–19). This normal marked rise in the protective AOE system in the late gestational fetal lung would imply that the very prematurely born would not only miss out on normal surfactant system development but would demonstrate incomplete maturation of their AOE defenses as well. This finding suggests that the prematurely born would be compromised under hyperoxic conditions and prone to develop rapid-onset O_2 toxicity, which might help explain why in human infants it is the more prematurely born who are most prone to develop early hyperoxic lung damage and progression to bronchopulmonary dysplasia (20).

However, an important caveat needs to be considered. The results of a large number of experimental O_2 toxicity studies have clearly indicated that baseline AOE levels are of lesser importance in determining resistance/susceptibility to O_2-induced lung damage (and lethality) than is the ability/inability to respond to hyperoxic exposure with an increase in pulmonary AOE activities. In general, neonatal animals have considerably lower AOE levels than adult animals, yet neonates demonstrate prolonged survival in hyperoxia in association with the ability to rapidly mount a protective AOE response to hyperoxic exposure, whereas the parent/adult animals manifest neither the ability to survive for more than 3–5 d in hyperoxia nor the ability to increase AOE activities during high O_2 exposure (1, 21). Thus, the premature born with lower baseline AOE protective capacity (and surfactant) than the full-term might still have the capability to increase their lung AOE activities (and surfactant levels) when challenged with hyperoxia and thereby not be as susceptible to pulmonary O_2 toxicity as their immature baseline levels might infer. Our experimental data support this concept because we consistently observed an ability for pulmonary AOE and surfactant responsiveness to hyperoxia in our prematurely born rat pups equivalent to the lung biochemical responsiveness to high O_2 observed in the full-term newborn pups. This biochemical adaptability of the premature lung helps to explain the key finding of our studies, *i.e.* that contrary to our original hypothesis, the prematurely born rat is *not* more susceptible to pulmonary O_2 toxicity than the full-term animal of this species.

A word needs to be said about the early deaths in our O_2-

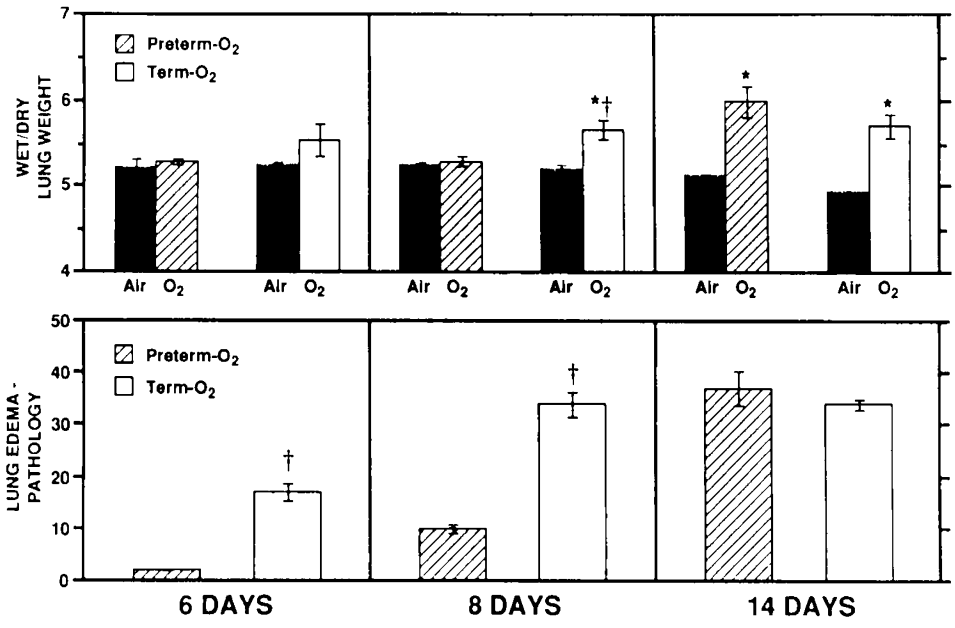

Fig. 2. Comparative evidence for pulmonary O_2 toxicity in prematurely born (*hatched bars*) and full-term (*white bars*) rat pups at 6, 8, and 14 d of >95% O_2 exposure. *Top*, Lung wet weight/dry weight values were mean ± SEM for 8–10 pups/O_2 group. The respective air group values (*black bars*) are also illustrated here. *Bottom*, Lung edema by microscopic pathologic analysis (%) was number with intraalveolar edema fluid per total number of coded lung sections examined (24–31/O_2 group). *Bars* indicate SEM (two observers). None of the lung sections in the air control groups were found to have edema fluid. *, $p < 0.01$ for O_2 group *vs* respective air group (for lung wet weight/dry weight); †, $p < 0.05$ or less for O_2-preterm *vs* O_2-term group.

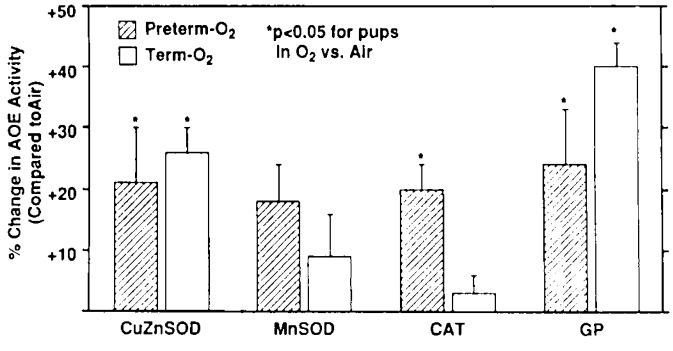

Fig. 3. Comparative pulmonary AOE responses of prematurely born (*hatched bars*) *vs* full-term (*white bars*) rat pups to 72 h of hyperoxia (>95% O_2). AOE activities were calculated as activity units/mg DNA and are expressed here as percentage change (means ± SEM) in O_2-exposed compared with air-exposed lung AOE values for eight samples per O_2 group. *CuZnSOD*, copper-zinc superoxide dismutase; *MnSOD*, manganese superoxide dismutase; *CAT*, catalase; *GP*, glutathione peroxidase. *, $p < 0.05$ or less for O_2 group *vs* respective air group.

exposed preterm rat pups (18% at 48 h in >95% O_2). We do not believe these deaths can be attributed to O_2 toxicity for the primary reason that our room air controls had much higher mortality rates at 24 and 48 h than the O_2-exposure group. We believe the early deaths are more likely because of the >25% lower lung surfactant in the preterm *versus* the term newborns. O_2 exposure actually enhanced the early survival of our preterm pups, which may be similar to the human situation in which prematurely born infants would die during the first 24 to 48 h of life without oxygen (ventilatory) support. Thus, we are of the opinion that Figure 1B probably is more representative of comparative O_2 tolerance of the preterm and term rats than Figure 1A is.

Because the lung biochemical responses of both our O_2 groups were essentially similar and the rates of lung cyanide-resistant O_2 consumption (a reflection of O_2 radical production) were not different between the two groups at 6-d exposure time, we cannot at this time explain why the premature rats actually had transiently improved survival and decreased evidence of O_2 toxicity (pulmonary edema) *versus* full-term newborn rats. Although it is possible that before d 6 of O_2 exposure the premature pups in O_2 could have had comparatively lower cyanide-resistant O_2 consumption, it seems unlikely that from d 7 to 9, when their survival rate was elevated, that they would suddenly manifest reduced lung cyanide-resistant O_2 consumption compared with the O_2 term pups.

To our knowledge, this is the first report of the ability of a premature altricial animal to show both relative hyperoxic tol-

Table 2. *Comparative lung AOE and surfactant content responses to 6 d of hyperoxic exposure**

Group	CuZnSOD	MnSOD	CAT	GP	DSPC	TPL
Premature rats—air	44.9 ± 1.7	1.27 ± 0.08	124 ± 4	0.25 ± 0.01	3.41 ± 0.37	17.3 ± 0.9
Premature rats—O_2	56.1 ± 2.4† (↑25%)	1.48 ± 0.20 (↑16%)	181 ± 7† (↑46%)	0.46 ± 0.01† (↑87%)	4.85 ± 0.29† (↑42%)	20.4 ± 0.6† (↑18%)
Newborns—air	50.1 ± 1.8	1.19 ± 0.06	123 ± 7	0.28 ± 0.01	3.22 ± 0.23	17.6 ± 0.6
Newborns—O_2	66.4 ± 1.1† (↑33%)	1.52 ± 0.16 (↑27%)	165 ± 8† (↑34%)	0.52 ± 0.01† (↑85%)	4.77 ± 0.59† (↑48%)	19.3 ± 1.4 (↑9%)

* Values are means ± SEM for 8 to 10 samples/group for AOE and 14 to 15 samples/group for DSPC and TPL. AOE enzyme activity calculated as activity units/mg DNA; DSPC and TPL calculated as mg/g lung. CuZnSOD, copper-zinc superoxide dismutase; MnSOD, manganese superoxide dismutase; CAT, catalase; GP, glutathione peroxidase.

† $p < 0.05$ for O_2 group *vs* respective air group.

erance and an appropriate biochemical protective response to prolonged hyperoxic challenge. For precocious animal species, Sosenko and Frank (22) earlier reported that prematurely delivered guinea pigs had markedly improved hyperoxic tolerance compared with full-term newborns and that (unlike term newborns) premature guinea pig pups were capable of mounting elevated AOE and surfactant responses to hyperoxic challenge. (Note: Altricial species are born in a very immature dependent state, with a morphologically immature lung and CNS, whereas precocious species deliver newborns with full body hair, eyes open, the ability to thermoregulate normally, and a structurally mature lung and CNS.) Our findings in prematurely born rat pups are considerably different than previous studies in prematurely delivered altricial animals of other species. Preterm rabbit pups (d 29 of a normal 32-d gestation) tolerate hyperoxia poorly compared with full-term newborn rabbits, with accelerated O_2 toxicity changes and a failure of the preterm animals to increase their AOE activities in response to hyperoxic challenge (23). Similarly, premature baboons (d 140 of a normal 180-d gestation period) reportedly fail to respond to lung-damaging periods in hyperoxia with elevated AOE activities (24).

Our present results for rats *in vivo* are consistent with some *in vitro* studies reported previously. Tanswell *et al.* (25) have found that cultured mixed fetal rat lung cells are capable of increasing AOE activities on exposure to hyperoxia (50% O_2). More recently, studies of 19-d fetal rat lung explant cultures from our laboratory have found that these fetal lungs are able to increase their AOE activities and their DSPC synthesis to an even more significant extent when growing in 90% O_2 compared with explants maintained in 21% O_2 (26). Loo *et al.* (27), however, found that cultured type 2 cells from fetal rat lung are susceptible to hyperoxic damage, with disruption of lamellar body formation and other signs of cytotoxicity, and that O_2 toxicity changes were even greater in 18- to 19-d fetal lung epithelial cells than 20- to 21-d fetal lungs. In contrast, their type 2 cells from newborn rat lungs were comparatively resistant to *in vitro* hyperoxic exposure (27). In comparing our present survival results in 21-d prematurely delivered rat pups with an earlier *in vivo* report by Tanswell *et al.* (28) using 21-d rat pups, we found that these investigators had much lower early survival rates than ours both in their high O_2 groups (~45%) and in their air group (only 6%) at 36 h of exposure. However, the pattern of the survival rate for the small number of preterms they tested in hyperoxia from 2 d to 14 d was essentially the same as our data, with most premature pups alive at 8–9 d of O_2 exposure and about half the pups surviving the full 14 d in high O_2. Only historic full-term newborn rat survival rates were used for comparative purposes in their study (28).

Thus, the differing abilities of the preterm rat compared with the premature newborns of other tested laboratory species (rabbits, baboons) to effectively protect their lungs from early life hyperoxic challenge leaves still open the critical question of whether the lung of the human premature infant can or cannot mount a protective antioxidant response to required high O_2 treatment.

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