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Premature Weaning of Rat Pups Results in Prolongation of Neonatal Tolerance to Hyperoxia

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ABSTRACT. Neonatal rats usually lose their marked tolerance to hyperoxia at about 1 mo of age. We examined the hypothesis that the marked dietary change that occurs at weaning might be important to this loss of O_2 tolerance. We, therefore, prematurely weaned rat pups at 15–17 d of age, expecting to find an earlier loss of O_2 tolerance. Surprisingly, the prematurely weaned rats showed consistently prolonged relative O_2 tolerance compared with normally weaned rats at all ages tested from 35–85 d of life. For example, when challenged with >95% O_2 exposure for 7 d, the composite survival rate of the prematurely weaned rats (at 35–85 d of age) was nearly twice that of the normally weaned group (83 of 107 = 78% *versus* 44 of 107 = 41%, $p < 0.01$). In the two experimental groups, nearly all comparative parameters examined were similar, including: *1*) growth rate; *2*) lung DNA, RNA, and protein; *3*) lung antioxidant enzymes and enzyme responses to hyperoxia; *4*) lung morphometry; and *5*) lung elastin and collagen content. Only serum corticosterone and triiodothyronine levels differed considerably in the two groups. We conclude that premature weaning has a very marked and sustained positive effect on the relative retention of O_2 tolerance in the growing rat. (*Pediatr Res* 29: 376–380, 1991)

Abbreviations

T_3, triiodothyronine
MDA, malonaldehyde

Neonatal animals of many species are quite resistant to pulmonary O_2 toxicity and survive much longer in hyperoxia (>95% O_2) than the adults of these species (1, 2). However, this relative tolerance to hyperoxia wanes at about 1 mo of life (3). The basis for this age-related loss of O_2 tolerance is not known.

We hypothesized that the chronologically related event of weaning and the rather drastic change in diet that occurs at weaning—from a high fat, low carbohydrate milk diet to a low fat, high carbohydrate rat pellet diet (protein contents equivalent)—could be associated with the subsequent loss of O_2 tolerance. We also hypothesized that maintaining weanling rats on a rat milk diet might extend their period of tolerance to hyperoxia. However, lacking a truly appropriate rat milk diet substitute, we elected instead to first try the opposite approach, *i.e.* to determine whether premature weaning of suckling rats (d 15–17) to a mashed pellet diet would result in an earlier or more profound loss of O_2 tolerance in comparison to rat pups normally weaned at 24–25 d of age. To our surprise, the prematurely weaned rat pups showed a prolonged relative O_2 tolerance to hyperoxia.

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MATERIALS AND METHODS

Animals. Newborn rat litters were obtained from our own approved breeding colony under veterinary supervision by the Division of Animal Care, University of Miami. Several litters were pooled at birth and redistributed to the dams in litter sizes of 10–12 pups. Dams were maintained on water and standard rat pellet diets (Rodent Laboratory Chow, no. 5001, Ralston Purina Co., St. Louis, MO) *ad libitum*, and on a 12 h:12 h dark/light cycle. Litters were normally weaned at 24–25 d of age to the rat pellet diet. Premature weanlings were removed from their dams at d 15–17 of age and put on a mashed pellet diet for several days and then whole rat pellets. Water was provided *ad libitum*. Daily growth changes were recorded for the two groups of weanlings. (The total protocol was preapproved by the University Animal Care and Animal Welfare Committee.)

Exposures to hyperoxia. Normally weaned and prematurely weaned rats were exposed together to >95% O_2 at various ages between 30 and 85 d of life. Exposures to O_2 were conducted in 3.5-ft^3 exposure chambers constructed from modified clear plastic nursery isolettes (model 86; Air Shield, Hatboro, PA). Exposure conditions were carefully controlled and monitored (96–98% O_2, <0.5% CO_2, 24–27°C, 50–75% humidity) with gas analyzers and in-chamber thermometer-hygrometers. All O_2 exposures were continuous, except for 10–15 min daily when the chambers were opened to change the bedding and supply fresh food and water. Exposures were for 72 h or for 7 d. O_2-induced lung damage was assessed by comparative survival, pleural fluid accumulation, lung wet/dry weights, lung weight/body weight ratios, and gross and light microscopic pathology.

Biochemical and blood studies. Blood from anesthetized rats (pentobarbital, 40 mg/kg, intraperitoneally) was collected from the inferior vena cava, and the serum levels of T_3 and corticosterone were measured by RIA using specific RIA kits with accompanying instructions (T_3, Cambridge Technology, Inc., Cambridge, MA; corticosterone, Radioassay Systems Laboratories, Carson, CA). All blood was collected between 1000 and 1200 h. Serum was frozen at −70°C before analysis.

Lung biochemical parameters were determined on rats exsanguinated by cutting the great vessels in the abdomen after deep pentobarbital anesthesia. The lungs were then rapidly perfused free of blood via the pulmonary artery with cold isotonic phosphate buffer (0.1 M potassium phosphate, 0.15 M KCl, pH 7.4). After the lungs were dissected free of nonpulmonary tissues, they were weighed and homogenized in cold hypotonic phosphate buffer (0.005 M potassium phosphate, pH 7.8) (10:1; vol/wt) in a polytron (Brinkman Instruments Co., Westbury, NY), and subsequently assayed for DNA (4), RNA (5), and protein content (6), and for the antioxidant enzymes superoxide dismutase (7), catalase (8), and glutathione peroxidase (9) using standard spectrophotometric assays. Purified reference standards for all these assays were obtained commercially (Sigma Chemical Co., St. Louis, MO).

Hydroxyproline values were determined in perfused lungs by the acid hydrolysis spectrophotometric method of Woessner (10).

Elastin was determined by the spectrophotometric method of Naum and Morgan (11).

We also assayed the lungs for lipid peroxidation products by using the MDA assay of Ohkawa *et al.* (12). This assay involved mixing 0.3 mL of lung homogenate, 0.2 mL of 8.1% SDS, and 1.5 mL of 20% acetic acid (adjusted to pH 3.5 with NaOH) with 2.0 mL of aqueous thiobarbituric acid. After 60 min of heating at 95°C, the solution was cooled, mixed vigorously with 5.0 mL of *n*-butanol, and subjected to centrifugation (2000 × *g* for 15 min). The absorbance of the upper organic layer was determined at 532 nm and compared with a standard curve of tetraethoxy propane reagent.

Microscopic studies. Lungs from killed rats of various ages were inflated with 10% buffered formaldehyde via tracheal cannula at a constant pressure of 20 cm H_2O (fixative). Lung volumes were determined by water displacement immediately after inflation and again just before sectioning 48 h later to correct for shrinkage. Similarly oriented sections of each lobe were prepared for hematoxylin and eosin staining and subsequent evaluation by light microscopy. For the morphometric studies, coded slides were examined at ×450 with an integrating eyepiece with a simple square-grid pattern (square grid with five horizontal lines and 25 intercept bars, model cPLW ×10/18 eyepiece, Zeiss Optical, Thornwood, NY). A minimum of 30 lung fields from each of three different lung sections per animal were examined.

To calculate the mean linear intercept (Lm, the average distance between alveolar walls), the formula $Lm = n \cdot L/\epsilon i$ was used, where n is the number of grid lines counted, L is the length of line, and ϵi is the sum of alveolar intercepts. To calculate the internal surface area (ISA), we used the formula $ISA = 4 \cdot V/Lm$, where V is the shrinkage-corrected lung volume by water displacement. The percent air space was determined by dividing the number of "hits" (intercept bars) falling on air spaces by the total number of intercept bars falling on lung tissue plus air spaces. All formulas and counting methods used are derived from Weibel (13).

Statistical analysis. For comparison of biochemical differences between the two groups of weanling rats, unpaired *t* test analysis was done. For comparing values for the two hyperoxic groups *versus* air control values, one-way analysis for variance was done followed by Duncan's multiple range test. Survival rates were compared statistically by χ^2 testing (14). For all statistical tests, a $p < 0.05$ value was considered to represent a significant difference between the compared values.

RESULTS

There were no differences in growth rate for the prematurely weaned *versus* the normally weaned rat pups, nor were lung weight/body weight ratios different at any chronologic time point. Similarly, lung protein, DNA, and RNA content and RNA/DNA ratio were longitudinally similar in the two groups of weanlings (Table 1).

Figure 1 illustrates the comparative survival data for the normally weaned and prematurely weaned rats exposed to hyperoxia (>95% O_2 × 72 h) at the indicated age intervals. At all ages tested, consistently increased tolerance to hyperoxia was observed in the prematurely weaned group of rats [composite 72-h survival rates = 104 of 199 (52%) normally weaned *versus* 161 of 198 (81%) prematurely weaned; $p < 0.01$].

Table 2 shows comparative pathologic changes in the hyperoxic survivors of the two experimental groups, indicating lesser evidence of hyperoxic lung damage in the prematurely weaned rat group. Qualitative assessment of coded slides similarly indicated less common and less diffuse O_2 toxicity changes in the prematurely weaned animals. Whereas both groups had prominent perivascular-peribronchiolar edema, areas of intraalveolar edema or hemorrhage were seen almost exclusively in the O_2-exposed normally weaned rat lungs.

Lung MDA levels, reflective of lipid peroxidation, were also significantly higher in the more O_2 susceptible normally weaned group than the more O_2 tolerant prematurely weaned group. Values after 72 h in >95% O_2 (age 40 d, $n = 4$ per group) were 0.032 ± 0.003 nmol MDA/g lung (air controls), 0.039 ± 0.006 (prematurely weaned), and 0.061 ± 0.017 (normally weaned); $p < 0.05$ for normally weaned in O_2 *versus* both other groups.

That the relative protection from O_2 toxicity manifested in the prematurely weaned group (Fig. 1) does not merely reflect a delay in the onset of severe O_2 toxicity progressing to lethality is evident from the survival results in Figure 2. With hyperoxic exposure extended to 7 d (>95% O_2), the prematurely weaned animals still demonstrated double the survival rate of the normally weaned group (78 *versus* 41%).

We were unable to explain the difference in survival rates on the basis of disparate lung antioxidant enzyme responses to hyperoxia. Baseline antioxidant enzyme levels (superoxide dismutase, catalase, and glutathione peroxidase) were similar in the two experimental groups; Figure 3 shows the similar elevation in lung enzymes in both groups of weanlings during O_2 exposure at age 40 d and an essentially similar lack of any significant antioxidant enzyme response to >95% O_2 exposure at age 60 d.

Because of our previous experience with altered lung structural development producing improved O_2 tolerance in castrated weanling rats (15), we examined comparative morphometry in the two present experimental groups of animals. No altered lung structural changes in the two groups of weanling rats were noted (data not shown). Similar specific lung volumes, mean airspace size, internal surface area for respiratory exchange, and internal surface area per 100 g body weight were present.

Similarly, because elastin and collagen deposition are actively ongoing processes around the time of weaning in the rat (15–17), we compared lung connective tissue development in our two experimental groups. No difference in collagen and elastin content in the lungs of the two weanling rat groups was observed. Hydroxyproline and elastin concentration (mg/g dry lung wt) at age 52 d were 8.87 ± 0.52 and 8.76 ± 1.10, and 68.2 ± 12.7 and 62.7 ± 13.6, respectively, for the normally and prematurely weaned animals ($n = 6$ per group).

Finally, because serum levels of corticosterone and T_3 are low in early postnatal life but begin to rise rapidly toward the time

Table 1. *Comparative lung biochemistries of prematurely weaned and normally weaned rat pups at various ages**

	Lung protein	Lung DNA	Lung RNA	Protein/DNA	RNA/DNA
Age 28 d					
Normally weaned (15)	55.7	5.95	2.71	9.37	0.463
Prematurely weaned (15)	59.0	5.89	3.17	10.02	0.540
Age 42 d					
Normally weaned (9)	104.8	8.13	3.92	12.91	0.485
Prematurely weaned (9)	95.7	8.51	4.28	11.16	0.503
Age 75 d					
Normally weaned (5)	113.6	9.77	5.15	11.57	0.528
Prematurely weaned (5)	113.0	10.6	5.31	10.66	0.501

* Values are mean total lung contents (mg) at ages indicated for (*n*) samples. No differences between groups at any age are significant at $p < 0.05$ level.

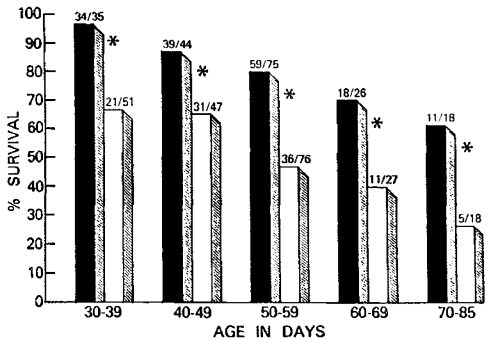

Fig. 1. Comparative survival rates for the prematurely weaned and normally weaned rat pups at the age intervals indicated. At all ages tested, the prematurely weaned group (*solid bars*) had statistically significant higher survival rates than the normally weaned group (*open bars*) during exposure to >95% O_2 for 72 h (*$p < 0.05$–0.01).

of weaning and both may affect lung growth and development (18, 19), we compared serum hormone levels in our two groups of weanling pups (Fig. 4). Corticosterone showed a large rise in serum level about a week earlier than normal in the prematurely weaned animals, and serum levels remained comparatively elevated to d 45. The opposite effect of premature weaning occurred with T_3, with continued relative depression of serum T_3 until d 45 compared with the normally weaned rat hormone levels.

DISCUSSION

One of the more intriguing unsolved mysteries of O_2 toxicity is the age-related pattern of tolerance to hyperoxia and progressive loss of O_2 tolerance by neonatal animals beginning at about 1 mo of age (1, 3, 20, 21). Before 1 mo of age, nearly 100% of neonatal rats will survive prolonged exposure to >95% O_2, yet by 2 mo of age <25% will survive similar O_2 challenge and the survivors will manifest severe O_2-induced lung damage. We and others have been able to associate the neonatal animal's tolerance to high O_2 with the rapid biochemical responsiveness of the immature animal's lung to hyperoxia, *i.e.* the neonatal lung manifests a marked increase in its antioxidant enzyme protective system shortly after the onset of exposure to high O_2 (2, 20, 21). This protective biochemical response is not seen in adult animals, which survive poorly in hyperoxia (2, 20, 22). Recently, these findings were reinforced with the demonstration of a rapid pretranslational induction by hyperoxia of pulmonary antioxidant enzyme mRNA in neonatal rats, but no similar effect on enzyme mRNA in simultaneously O_2-exposed adult animals (23).

We have previously approached the question of why neonatal animals lose their O_2 tolerance at about 1 mo of age by examining the effects of castration at 20 d of age, just before the pubertal surge in sexual hormones occurs in rat pups. Male castration, which served to abrogate the serum testosterone hormone surge at approximately 1 mo of age, both altered subsequent lung growth and prolonged relative O_2 tolerance (15). The significantly enlarged lung volumes and mean lung airspace sizes were determined to be the most likely factors related to the prolongation of relative O_2 tolerance in the castrated male rats. In turn, the altered hormonal milieu (increased serum growth hormone and glucocorticoid levels plus decreased testosterone) was postulated to have caused the altered lung growth pattern in the castrated rats (15).

Weaning is a key event occurring at approximately 1 mo of age in the rat because of the rather drastic change in dietary composition it entails and the possible effect of altered diet on the developing lung and its susceptibility to hyperoxia. Our prematurely weaned rats experienced a sudden switch from a milk diet low in carbohydrate (3%) and high in fat (12.5%, primarily saturated fatty acids) 8–10 d before the control weanlings were switched from milk to the high carbohydrate (25%) and low fat (4.5%, primarily unsaturated fatty acids) rat pellet diet (24, 25). The dietary change at weaning results in rapid increases in enzymes involved in lipogenesis and concurrent rapid decreases in enzymes involved in glucogenesis and fatty acid oxidation (26–28). However, as opposed to these metabolic changes in the liver, fatty tissue, and gastrointestinal tract, no information is available about dietary-induced lung metabolic, enzymic, or growth changes specifically associated with weaning. Wide changes in hormonal levels also occur around the time of weaning, with rapidly decreased serum glucagon, increased insulin, and a rapid increase in serum glucocorticoid hormone (18, 29, 30). Plasma T_3 also rises just before the time of normal weaning in rats (31). Other hormonal factors that normally increase in rats around the normal time of weaning include growth hormone (32) and somatomedin C (33). β-Adrenergic receptors in the rat lung markedly increase between d 15–28, and this change in receptor density is related to the normal rise in serum T_3 levels at this time (34). The rise in serum growth hormone levels at approximately 20 d of age is also dependent upon the normal rise of T_3 and the rise in glucocorticoid hormones at this time (35). These three hormones, which each rise around the time of weaning, have known lung growth effects (36, 37).

The premature switch (age 15–17 d) to a weanling diet in our studies resulted in a dramatic retention of relative tolerance to hyperoxia over the next 2 mo of life. The mechanism(s) of our observed protective effect associated with premature weaning remains unclear. We noted that the important lung antioxidant enzyme responses to hyperoxia were similar in the two groups of weanling animals. No meaningful differences in lung structure (morphometry) were found in comparing our two groups of weanling rats at several later ages. Similarly, lung elastin and collagen deposition were similar in the growing lungs of the two experimental groups. The very striking differences in the patterns of serum T_3 and corticosterone levels are intriguing, but just how they might have affected O_2 tolerance per se is difficult to define. Elevated corticosteroid levels by exogenous means actually tend to exacerbate the course of O_2 toxicity (38, 39). Conceivably, the suppressed serum T_3 activity in the prematurely weaned pups was associated with depressed O_2 consumption and reduced O_2 radical production (not measured), but by 45 d of life T_3 levels were equivalent in the two experimental groups and relative O_2 tolerance persisted in the prematurely weaned rat group.

There is good evidence that the lungs will rather rapidly assume a fatty acid composition that reflects the dietary fatty acid profile and much theoretical but less experimental evidence that a more saturated fatty acid diet has a relative protective effect against O_2 toxicity (40–42). This may be because polyunsaturated fatty acids are prone to O_2 free radical attack and lipid peroxidation, but

Table 2. *Comparative pathologic changes in two experimental groups (n) exposed to hyperoxia (>95% O_2 for 72 h)*

Group	Pleural fluid (mL)	Lung wt/body wt	Lung wet/dry wt
Air controls	0.06 ± 0.06 (10)	0.433 ± 0.107 (12)*	4.96 ± 0.15 (8)*
O_2 normally weaned	2.85 ± 3.09 (11)†	1.015 ± 0.402 (13)	5.54 ± 0.03 (9)
O_2 prematurely weaned	0.87 ± 0.13 (11)	0.744 ± 0.119 (13)‡	5.34 ± 0.26 (9)‡

* $p < 0.01$ *vs* both O_2 groups.
† $p < 0.05$ compared with both other groups.
‡ $p < 0.05$ *vs* other O_2 group.

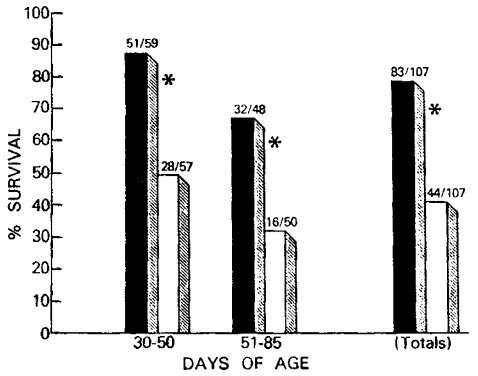

Fig. 2. Comparative survival rates when >95% O_2 exposure was extended to 7 d. At both younger (30–50 d of age) and older ages (51–85 d of age), the prematurely weaned group of rats (*solid bars*) had statistically significant improved survival rates compared with the normally weaned animals (*open bars*) (*$p < 0.01$). The prematurely weaned rats also had significantly improved total survival rates in >95% O_2 for 7 d (78 *vs* 41%).

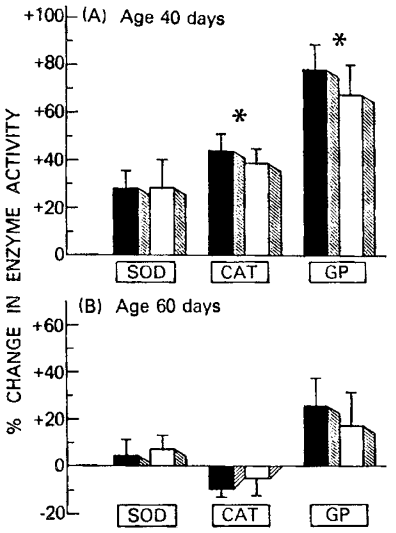

Fig. 3. Changes in lung antioxidant enzyme activity during exposure to >95% O_2 for 72 h. The values were calculated as activity units/mg DNA and are plotted here as % change compared with air control enzyme activity levels. *A*, at age 40 d, both experimental groups—the prematurely weaned (*solid bars*) and the normally weaned rats (*open bars*)—manifest similar increases in lung superoxide dismutase (*SOD*), catalase (*CAT*), and glutathione peroxidase (*GP*) activities compared with air control levels (the zero value) (*$p < 0.05$, $n = 6$ per group, mean + SEM bars). *B*, by age 60 d, both experimental groups show a similar lack of any significant antioxidant enzyme increase during O_2 exposure ($n = 6$ per group, mean ± SEM bars). Air control enzyme values: SOD 28.5 ± 2.2 units/mg DNA; CAT 573 ± 16; and GP 1.58 ± 0.14.

saturated fatty acids are resistant to lipid peroxidation damage (43). We do not believe that the improved survival of the prematurely weaned animals was related to their lung fatty acid profile. Although these rat pup lungs might be expected to have a more unsaturated fatty acid composition (due to pellet *versus* milk diet) before the normally weaned pups, their O_2 tolerance was obviously not impaired by this. Moreover, in the later ages tested, both experimental groups might be expected to have attained very similar lung lipid profiles reflecting the same rat pellet (polyunsaturated fat-rich) diet.

We conclude that the premature weaning of rat pups results in a marked prolongation of relative tolerance to hyperoxia. After examining all the comparative parameters tested, we do not yet have a satisfactory explanation for the effectiveness of early weaning on extending the normal period of neonatal O_2 tolerance.

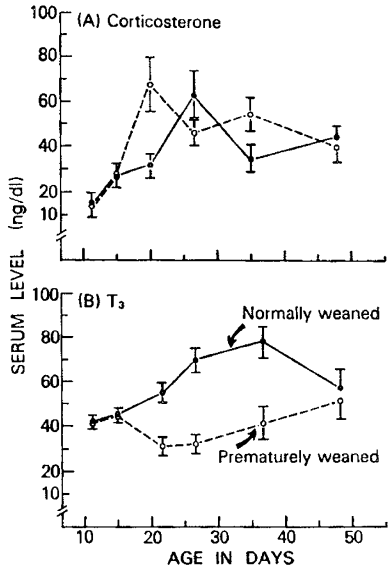

Fig. 4. Comparative serum hormone levels, ages 12 to 50 d, in the two groups of experimental animals (mean ± 1 SD; $n = 3$ per time point). *A*, corticosterone, showing earlier rise and sustained elevation in prematurely weaned (O) rats compared with the normally weaned animals (●). *B*, T_3, showing sustained depression of T_3 in the prematurely weaned rats (O) *vs* the normally weaned group (●). Values for both hormones in both groups become equivalent by age 50 d.

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