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Prenatal Thyroid Releasing Hormone and Thyroid Releasing Hormone Plus Dexamethasone Lessen the Survival of Newborn Rats during Prolonged High O_2 Exposure

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ABSTRACT. Newborn rats prenatally treated with TRH or the combination of TRH+DEX have lower lung antioxidant enzyme activities at birth than control newborns but are able to induce an adaptive antioxidant enzyme response to hyperoxic exposure of similar or even greater magnitude compared to O_2 control offspring. Because of this greater antioxidant enzyme response, we hypothesized that the hormonally pretreated newborns might demonstrate superior tolerance to prolonged high O_2 exposure. However, when placed in >95% O_2 at birth, the survival rates were consistently lower in the TRH- and TRH+DEX-treated pups at all time periods in hyperoxia from 9 d [control = 74 of 92 (80%); TRH+DEX = 32 of 47 (68%); TRH = 29 of 48 (60%); $p < 0.05$] to 14 d [control = 43 of 92 (47%); TRH+DEX = 11 of 47 (23%); TRH = nine of 48 (19%); ($p < 0.05$)]. Other evidence of poorer O_2 tolerance in the prenatal hormone-treated pups included a greater incidence of intraalveolar edema and elevated lung conjugated dienes, an index of lipid peroxidation, at 3, 5, and 7 d of O_2 exposure. There was also a persistent elevation in 3,5,3′-triiodo-L-thyronine and thyroxine serum levels in the 10-d-old TRH-treated offspring. We conclude that prenatal TRH treatment, possibly working through the secretion of 3,5,3′-triiodo-L-thyronine and thyroxine, has some important lasting postnatal effect (not completely reversed by dexamethasone) that predisposes newborn rats to greater O_2 radical-induced lung sequelae of prolonged hyperoxic exposure. (*Pediatr Res* 32: 407–411, 1992)

Abbreviations

TRH, thyrotropin releasing hormone
DEX, dexamethasone
AOE, antioxidant enzyme
Lm, mean chord length (= mean air space diameter)
ISA, internal surface area
T_3, 3,5,3′-triiodo-L-thyronine
T_4, thyroxine

Just as the surfactant system of the fetal lung develops in the final part of gestation, the normal development of the pulmonary AOE system (superoxide dismutase, catalase, and glutathione peroxide) has recently been shown to chronologically parallel the development of the surfactant system (1–5). Experimental evidence also suggests that these two biochemical systems share some of the same hormonal regulators as well. The administration of DEX to pregnant rats in late gestation produces acceleration in both surfactant and AOE system development in their fetal offspring (6). However, whereas the administration of T_3 or TRH to pregnant rats in late gestation produces substantial increases in fetal lung surfactant development, their effect on the AOE system is opposite to what is seen for surfactant, with the treated fetal offspring demonstrating a developmental delay in the pulmonary AOE (7, 8). The addition of prenatal DEX to TRH produced an expected synergistic increase in surfactant with an unexpected enhanced negative effect on AOE system maturation (8). Despite lower AOE at the time of birth, newborn rats prenatally treated with either TRH or the combination of TRH plus DEX were surprisingly able to mount a normal protective AOE response during hyperoxic exposure that was of similar or even greater magnitude compared with control O_2-exposed offspring (8).

To conclude the above series of hormonal studies on the developmental regulation of the lung AOE system, we hypothesized that newborn rats prenatally treated with either TRH or the combination of TRH plus DEX would survive as well as, if not better than, control newborn rats when exposed postnatally to a high O_2 challenge. Our hypothesis was based primarily on the greater AOE response to hyperoxia in the TRH and TRH+DEX newborns. When we consistently found that the results of our hyperoxic survival studies failed to support this hypothesis, we explored a variety of parameters of pulmonary O_2 toxicity in the rapidly growing lung to try to understand the decreased hyperoxic survival of the hormonally exposed newborns.

MATERIALS AND METHODS

Animals and treatment. Sprague-Dawley albino rats originally obtained from Charles River Laboratories (Wilmington, MA) and maintained in the Animal Care Facilities of the University of Miami School of Medicine under veterinary supervision were used. Breeding was accomplished by placing male and female animals together overnight, checking for sperm-positive vaginal smears the following morning, and considering the midpoint of the cohabitation period as the onset of pregnancy. The timed-pregnant rats were maintained on water and standard rat pellet diets (Rodent Laboratory Chow, Ralston Purina Co., St. Louis, MO) *ad libitum* and kept on a 12-h light/dark cycle.

At 48 h before expected spontaneous delivery of full-term (d 22 of gestation) offspring, pregnant dams were randomly assigned to either a control group, a TRH treatment group, or a TRH+DEX treatment group; for studies examining TRH treatment alone, TRH (Bachem Inc., Torrance, CA) was administered

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s.c. to the pregnant dams as a loading dose (25 μg/kg) and by s.c. implantation of an Alzet osmotic minipump (Alza Corp., Palo Alto, CA) through which continuous TRH was administered (100 μg/kg/d). These doses were chosen based on previous work by Rooney *et al.* (9) and Ikegami *et al.* (10). The control groups received an equivolume s.c. injection of saline, as well as "sham" surgery under the same anesthesia (ketamine:xylazine anesthesia, 90 mg/kg:10 mg/kg; Ketalar, Parke-Davis, Morris Plains, NJ and Rompun, Paynet Division-Cutter Labs, Shawnee, KS). For the studies involving TRH+DEX treatment, TRH was administered as described above, and in addition DEX (dexamethasone sodium phosphate, Henry Schein, Inc., Woodbury, NY) was administered at 48 and 24 h before delivery at a dose of 0.4 mg/kg intraperitoneally. The control groups received an equivalent dose (0.5 mL/100 g) of intraperitoneal saline. After prenatal hormone or saline treatment, rat newborns were obtained via normal parturition within 6 h of the beginning of delivery of the first pup. The newborn pups of several equivalently treated litters were first pooled and then redistributed to the equivalently treated newly delivered dams. Dams plus 10–12 pups/litter were randomly assigned to either a hyperoxic exposure (>95% O_2) or room air exposure group.

Exposures to hyperoxia were conducted in 3.5-ft^3 clear plastic exposure chambers adapted from regular infant isolettes (model 86; Air Shields, Hatboro, PA). The O_2 and CO_2 levels were monitored at frequent intervals with Beckmann model OM-11 and LB-2 gas analyses (Beckman Instruments, Inc., Schiller Park, IL). The O_2 concentration was maintained at 96–98%, and CO_2 at less than 0.4%. The temperature in the chambers was 24–26°C (in-chamber thermometers), and the humidity was 50–70% (in-chamber hygrometers). The chambers were opened daily (10–15 min) to provide fresh food, water, and cages, to weigh the rat litters, and to interchange mothers between litters exposed to O_2 and room air to avoid O_2 intoxication in the nursing dams. The offspring were either maintained in hyperoxia for 14 d for survival studies or killed (with an overdose of pentobarbital) after 3, 5, 7, or 10 d of either hyperoxia (>95% O_2) or room air exposure. Their lungs were either used for biochemical analyses or were inflated *in situ* through a tracheal catheter with 10% buffered formalin (20 cm H_2O pressure) and stained with hematoxylin and eosin for microscopic studies and assessment of intraalveolar edema. The total experimental protocol was preapproved by the University of Miami Animal Care and Animal Welfare Committee.

Lung analyses. Lung lipid peroxidation was assayed by conjugated diene measurements (11). After an overdose of intraperitoneal pentobarbital, a midline abdominal incision was made, the aorta was severed to exsanguinate the animal, and the chest cavity was opened in the midline. The lungs were rapidly perfused with cold water via the pulmonary artery after removing the left atrial appendage to facilitate draining. The dissected lungs were then weighed and homogenized (Brinkmann Polytron Homogenizer) in isotonic phosphate buffer containing 0.001 M EDTA. After centrifugation, the homogenate supernatant was extracted with 2 volumes of 45°C methanol:chloroform (1:2) two times, then once with water:methanol:chloroform (1:1:2). After the last centrifugation, the methanol-water upper phase was aspirated off and a 2-mL aliquot of the chloroform extract was dried in a water bath at 40–50°C under nitrogen. The lipid residual was redissolved in 3 mL of heptane, and the absorption spectrum at 223 nm was recorded against a heptane blank. Results are expressed as absorbance units per g wet lung (11).

Lung elastin was assayed by the spectrophotometric method of Naum and Morgan (12). After homogenizing the lungs in 0.15 M NaCl and centrifugation, 1.0 mL of 5 M guanidine HCl, pH 7.0, was added to the pellet. Guanidine extraction continued for 24 h at room temperature in a shaking water bath. After centrifugation and a second 24-h extraction with guanidine, the residue was suspended in 1.0 mL H_2O and then autoclaved at 15 lb pressure for 45 min. After centrifugation, the precipitate was mixed with 1.0 mL H_2O and 1.0 mL elastase solution (0.1 mg purified elastase in 0.1 M veronal acetate buffer, pH 8.8) and incubated for 30 min at 37°C in a shaking water bath. Aliquots were then assayed for protein (elastin) content (13). Purified standards of elastin (Sigma Chemical Co., St. Louis, MO) were treated as above for generation of a standard assay curve.

Serum hormone assay. From randomly selected prenatally TRH-treated and control newborns and 10-d-old offspring, hormone assays for serum T_3 and T_4 were done using specific RIA kits (Incstar Corporation, Stillwater, MN). The assay sensitivities for T_3 and T_4 were 0.138 nmol/L and ≤10.1 nmol/L, respectively.

Microscopic studies. For microscopic studies, lung inflation/fixation was performed as described above. Fixation was continued in formalin at room temperature for 48 h before determination of lung volume by water displacement (14) and sectioning. From all lungs, similarly oriented sections from similar portions of the left lung and the right middle and lower lobes were stained with hematoxylin and eosin and initially examined to eliminate any sections with evidence of inadequate preparation (atelectasis). No correction was made for tissue shrinkage. Light level morphometric assessment was done on coded slides for comparison of lung structural development (alveolarization) in air- and O_2-exposed neonatal animals from all experimental groups. We determined Lm, % air/% tissue space, ISA, and specific ISA (ISA/100 g body weight), using a standard integrating eyepiece with seven horizontal lines and 42 intercept bars (Zeiss Optical, Oberkochen, Germany). The coded slides were examined at ×400 magnification, with 30 random fields counted per slide. We counted the number of times the intercept bars fell on lung tissue per field and the number of times the lines were crossed by tissue septa per field. To calculate Lm (representing the average distance between air space walls, or mean air space diameter), we used the following formula: Lm = (length of line × no. of lines counted × no. of fields)/total no. of tissue intercepts, where the length of each line = 0.20 mm, number of lines = 7, and number of fields = 30 (15).

ISA represents the internal surface area of the lung available for respiratory exchange. ISA was calculated using the following formula: ISA = (4 × lung volume)/Lm, where lung volume is the determined postfixation lung volume (16). Percent air space was calculated as follows: % air space = Pa/(Pa + Pt) × 100, where Pa is the total number of intercept bars hitting air and Pt is the total number of intercept bars hitting tissue.

The presence of pulmonary edema was microscopically assessed by evidence of interstitial, peribronchial/perivascular, and intraalveolar edema in the coded sections of the O_2-exposed rat lungs. Pulmonary edema was also assessed by comparative wet-to-dry lung weights, using nonperfused lung lobes weighed before and after drying in an 80°C oven for 48–72 h to reach constant weight.

Statistical analysis. Survival rates of the treated *versus* untreated rat pups and assessment of intraalveolar edema were compared by χ^2 testing (17). For comparing biochemical values for the two hyperoxic groups with those of the two air control values, one-way analysis of variance was done followed by Duncan's multiple range test (17). For all statistical tests, a $p < 0.05$ value was considered to represent a significant difference between the compared values.

RESULTS

Physical characteristics. The influence of hormonal pretreatment and hyperoxia on body weight and lung weight were determined after 3, 5, and 7 d of hyperoxia in the offspring prenatally treated with TRH and TRH+DEX *versus* saline-control offspring. There were no differences in lung weight, body weight, or lung weight to body weight ratio in either the TRH- or TRH+DEX-treated offspring compared to the saline-control pups at these points in time (data not shown).

Survival data. The offspring prenatally treated with TRH demonstrated a significantly decreased survival rate compared to the control offspring from the 7th d onward in hyperoxia. The offspring prenatally treated with TRH+DEX demonstrated a significantly decreased survival rate compared to the control offspring from the 9th d onward in hyperoxia ($p < 0.05$ by χ^2 testing) (Fig. 1).

Lung analyses. Lung conjugated diene levels (an index of lipid peroxidation) after 3, 5, and 7 d of >95% O_2 for TRH, TRH+DEX, and control offspring are illustrated in Figure 2. The saline-treated offspring had only minimal increases in conjugated diene levels after 3, 5, and 7 d of hyperoxia. In contrast, the offspring prenatally treated with TRH had a 55% increase above the air TRH values at 3 and 5 d, with a 147% increase after 7 d of hyperoxia ($p < 0.05$). The offspring prenatally treated with TRH+DEX had approximately a 52% increase in lung conjugated dienes at 3 and 5 d of O_2 exposure, with a 65% increase at 7 d of hyperoxia ($p < 0.05$).

There were no differences found in lung elastin content between the offspring prenatally treated with TRH, TRH+DEX, or saline after a hyperoxic exposure (data not shown).

Hormone assays. Newborn offspring prenatally treated with TRH and saline-control offspring were found to have serum thyroid hormone levels that were unmeasurable, that is, below the sensitivity limits of the RIA used in this study.

The T_3 and T_4 serum levels in TRH-treated and control offspring after 10 d in >95% O_2 or room air exposure are shown in Table 1. Prenatal TRH treatment resulted in a consistent increase in T_3 and T_4 serum levels.

Microscopic studies. The comparative lung morphometry for TRH-, TRH+DEX-, and saline-treated offspring after 7 d of hyperoxia or room air exposure are illustrated in Figure 3. The poorer survival of the TRH and TRH+DEX offspring in hyperoxia was not accompanied by any difference in the degree of O_2-induced inhibition of normal lung structural maturation (alveolarization) occurring during hyperoxia. Compared to the air control values, the lungs of the TRH, TRH+DEX, and saline-control O_2-exposed offspring had similarly enlarged mean air space diameters (Lm) and similarly reduced lung surface area available for gas exchange per unit body mass (specific ISA). There were also no differences in % air space (76.51 ± 5.65, 73.90 ± 3.34, and 73.71 ± 2.81%) or lung volume/100 g (4.60 ± 0.88, 5.29 ± 0.85, and 4.92 ± 0.80) between the TRH, TRH+DEX, and saline-control O_2-exposed groups, respectively.

On light microscopic examination, all the O_2-exposed pups had evidence of perivascular/peribronchiolar edema present after 7 d of survival in O_2. This microscopic finding was further substantiated by wet/dry lung weights of the O_2-exposed offspring prenatally treated with TRH, TRH+DEX, and saline *versus* the offspring maintained in room air. However, there were no differences in wet/dry lung weights between the TRH (6.07 ± 0.71), TRH+DEX (6.14 ± 0.43), and saline-control (6.09 ± 0.15) offspring after 7 d of O_2-exposure (average air control was 5.45 ± 0.15). As evidence of more advanced O_2 toxicity, intraalveolar edema was present in 44% (28 of 63) of the TRH group lung sections ($p < 0.05$), compared to 16% (14 of 90) of the TRH+DEX lung sections and 14% (23 of 162) of the saline-control lung sections (Fig. 4). The similar increase in wet/dry weights in the O_2 groups suggests endothelial cell injury in all the groups. The intraalveolar edema results suggest different degrees of alveolar epithelial injury in the O_2 groups.

DISCUSSION

Many investigators have reported on the relative tolerance to equivalent hyperoxic challenges of the immature animal compared to the adult animal (18, 19). Because hyperoxia is presumed to upset the normal cellular oxidant-antioxidant defense equilibrium by producing a marked increase in O_2 free radical production, the most important factor in protection of the neonatal lung from O_2-induced lung injury appears to be the ability to respond rapidly to a hyperoxic challenge with an increase over the basal lung levels of the AOE (18–23). Because the TRH and TRH+DEX offspring had recently been found to exhibit the ability to respond very effectively to hyperoxia with an increase in their AOE (8), we hypothesized that they would have resisted O_2-induced severe lung damage and lethality at least as well as the control newborns exposed to hyperoxia. The comparative intolerance to hyperoxia that we consistently found in the newborns prenatally treated with TRH alone and TRH+DEX (Fig. 1), contrary to our original hypothesis, was quite unexpected.

In addition to the poorer survival in hyperoxia of the hormonally pretreated pups, we found compounding evidence of increased susceptibility to O_2 radical-induced lung damage in the

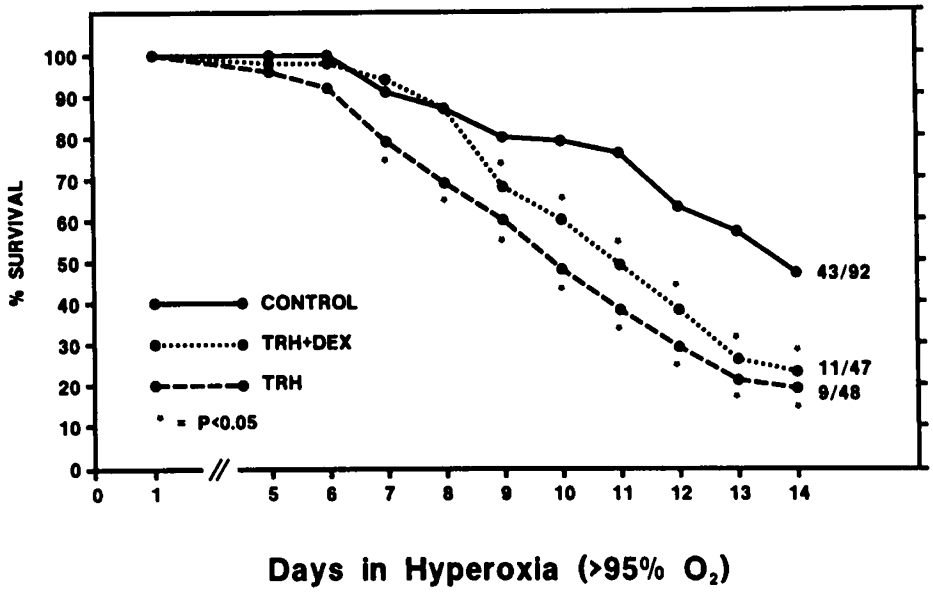

Fig. 1. Survival of prenatal TRH-treated, prenatal TRH+DEX-treated, and prenatal saline-treated (control) newborns in >95% O_2 for 14 d (n/n = number alive/number put in O_2). The O_2-TRH pup survival rate is significantly decreased compared to O_2 controls at all time periods from 7 to 14 d in hyperoxia. The O_2-TRH+DEX pup survival rate is significantly decreased compared to O_2 controls at all time periods from 9 to 14 d in hyperoxia (*, $p < 0.05$, χ^2).

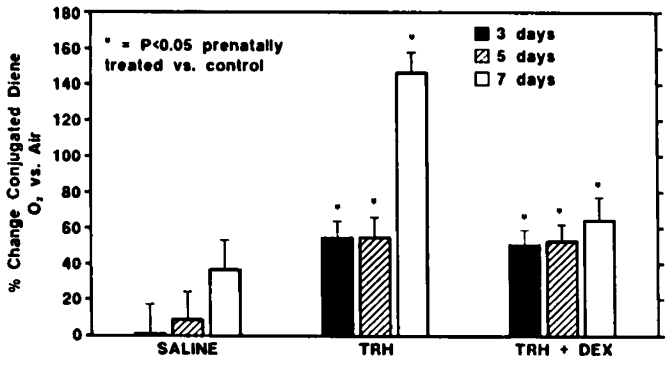

Fig. 2. Comparative lung conjugated diene response after 3, 5, and 7 d of >95% O_2 for TRH-, TRH+DEX-, and saline-treated offspring. Values are expressed as mean (% change) ± SEM in conjugated diene levels between O_2-exposed and air-exposed rat lungs (n = 3–4 litters per group; 10–14 samples per group). The offspring prenatally treated with TRH and TRH+DEX had a significant increase in conjugated diene levels compared to controls after 3, 5, and 7 d of hyperoxia (*, $p < 0.05$, prenatally treated *vs* control on respective days).

Table 1. *Serum T_3 and T_4 levels in TRH-treated and control offspring after 10 d in >95% O_2 or room air**

Treatment group	T_3 (nmol/L)	T_4 (nmol/L)
Air control	0.394 ± 0.147	15.61 ± 2.98
Air TRH	0.662 ± 0.230†	24.62 ± 5.50†
O_2 control	0.460 ± 0.135	17.91 ± 6.79
O_2 TRH	0.612 ± 0.107	22.39 ± 1.83
Air + O_2 control	0.424 ± 0.140	16.67 ± 5.01
Air + O_2 TRH	0.643 ± 0.188‡	23.65 ± 4.33‡

* Values are means ± 1 SD for five to nine samples in each group.
† Statistically significant with a $p < 0.01$ for air TRH *vs* air control.
‡ Statistically significant with a $p < 0.01$ for air + O_2 TRH *vs* air + O_2 control.

lipid peroxidation and intraalveolar edema data. The overall effect of lipid peroxidation is to decrease membrane fluidity, increase the "leakiness" of membranes to substances that do not normally cross it, and inactivate membrane-bound enzymes (11). Lipid peroxidation was indirectly measured in this study by assessing the formation of conjugated dienes (the oxidation of unsaturated fatty acid double bonds is accompanied by the formation of conjugated dienes). This method is known to have methodologic shortcomings [including the fact that the quantitation of conjugated dienes cannot be used as an absolute measure of *in vivo* lipid peroxidation (24)]; nonetheless, it provides generally acceptable comparative means of assessing hyperoxia-related lung damage and demonstrated a significant increase in the offspring prenatally treated with TRH and TRH+DEX compared to control offspring in O_2. Perivascular and peribronchiolar edema was detectable in all of the O_2-exposed groups, which was further substantiated by increases in wet/dry measurements after 7 d in >95% O_2. However, intraalveolar edema, which reflects severe O_2-induced damage to the epithelial plus endothelial cell barriers of the lungs, was markedly increased in the pups prenatally treated with TRH, again providing evidence of increased susceptibility to O_2 radical-induced lung damage in this treatment group especially.

To possibly further explain the unexpected reduced tolerance to hyperoxia in early life after prenatal treatment with TRH and TRH+DEX, we next focused on the alveolarization process that occurs between postnatal d 4 and 14 (25–27). Hyperoxia during this very organized phase of lung development results in inhibition of alveolar septation (28, 29). Therefore, if more severe morphometric alterations had been responsible for the poorer survival of the prenatally hormone-treated offspring, we would have expected to find more pronounced inhibition of alveolarization and surface area expansion. Because we did not find a greater increase in mean air space diameter and, most importantly, a greater decrease in specific ISA development in the hormone-treated offspring after hyperoxia, more pronounced morphometric alterations did not provide an explanation for their increased lethality in high O_2.

Elastin is a matrix protein which is integrally involved in the formation of new alveoli by septation (30–32). Hyperoxia is reported to inhibit normal elastogenesis in the developing lung (33, 34). Since meaningful differences in lung elastin content between the different O_2 groups were not found (nor reflected in morphometric changes), greater O_2 inhibition of elastogenesis cannot explain the disparate survival results between the hormone-treated and control pups in hyperoxia.

It has been previously shown in our laboratory that prenatal DEX treatment results in a significant degree of protection of newborn rats during hyperoxic exposures (35). The offspring prenatally treated with DEX exhibited no differences in morphometry or elastin content during O_2 exposure compared with control O_2 newborns, but a substantial decrease was found in

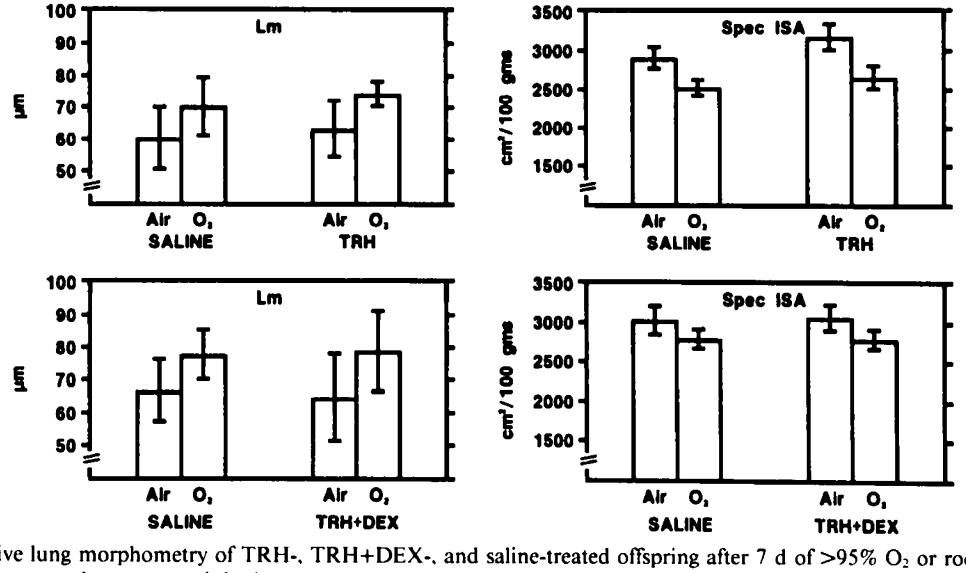

Fig. 3. Comparative lung morphometry of TRH-, TRH+DEX-, and saline-treated offspring after 7 d of >95% O_2 or room air exposure. *Spec ISA*, ISA available for gas exchange per unit body mass. Values are mean ± 1 SD for two to three litters per group; eight to 10 samples per group. The offspring prenatally treated with TRH, TRH+DEX, or saline demonstrated no difference in the morphometry of their lungs.

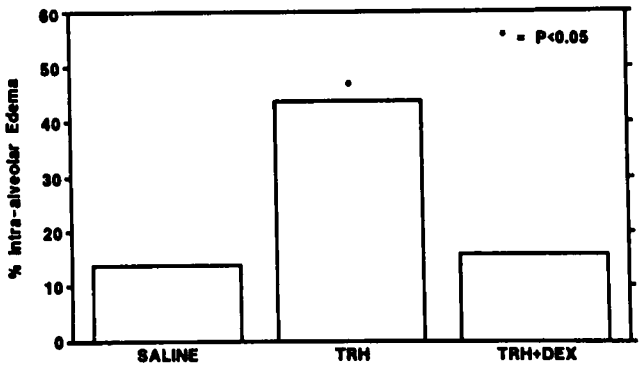

Fig. 4. Comparative incidence of intraalveolar edema in TRH-, TRH+DEX-, and saline-treated offspring after 7 d of >95% O_2 exposure. Lung edema by microscopic pathology represents the lung sections with intraalveolar edema fluid per number of lung sections examined (n = 2–3 litters per group). The offspring prenatally treated with TRH had a marked increase in incidence of intraalveolar edema (*, $p < 0.05$, χ^2).

conjugated diene levels as well as intraalveolar edema (results completely opposite from those found for TRH treatment in the present study). It would appear, therefore, from the combined findings of the DEX studies (35) plus the present TRH and TRH+DEX studies, that TRH may be the primary treatment agent responsible for compromising O_2 tolerance in the treated neonatal rats. The facts that the survival rates in the TRH alone group were lower than those in the TRH+DEX group and the conjugated diene levels (especially at 7 d) and incidence of intraalveolar edema reflected greater O_2 toxicity in the TRH alone group than in the combined treatment group would tend to support this notion.

A possible explanation for the detrimental effects of TRH treatment is that, because the maturation of the rat hypothalamic-pituitary axis normally occurs relatively late in the neonatal period (36–38), newborn rats during the period of O_2 exposure that we used are in a relative hypothyroid state (39). Despite previously showing increased T_3 and T_4 serum levels in TRH-treated *versus* control dams (8), we were unable to measure serum thyroid hormone levels in either the prenatally TRH-treated or control newborn pups using the most sensitive RIA commercially available. However, we were able to show significant elevations in T_3 and T_4 serum levels in the 10-d-old TRH-treated offspring. Thus, it may well be that our prenatally TRH-treated offspring had been made relatively hyperthyroid in the neonatal period, whereas the control offspring remained comparatively hypothyroid. This relative hyperthyroidism with resultant increased O_2 consumption (40), and presumably further increased O_2-radical production, could potentially exceed the detoxifying capacity of the antioxidant defense mechanisms and produce an increased susceptibility to O_2 toxicity.

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