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Can Maternal Vitamin E Supplementation Prevent Lung Hypoplasia in the Nitrofen-Induced Rat Model of Congenital Diaphragmatic Hernia?

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ABSTRACT

Recent studies suggest a role for antioxidants in the prevention of pulmonary hypoplasia associated with congenital diaphragmatic hernia (CDH). We studied the effects of vitamin E in the nitrofen-rat model of CDH. After an initial fast, timed-pregnant Sprague-Dawley rats were gavage-fed nitrofen at gestational day 11 (term is 22 d). On the same day, one group was given a s.c. injection of vitamin E in alcohol; a second group was given an injection of alcohol alone. A third group received no treatment (control). Fetuses were delivered on day 21, and static pressure-volume curves were measured by immersion. Lungs were analyzed for total DNA and protein content by standard methods. A total of 203 fetuses were studied. Of 151 nitrofen-exposed fetuses, 77% had CDH; 92% of these were right-sided. CDH was present in 82% of vehicle-treated fetuses and 71% of vitamin E–treated fetuses ($p = 0.17$). Nitrofen-exposed fetuses not only were smaller than control fetuses but also had disproportionately smaller lungs and poorer lung function, even when CDH was absent; however, lung function was worse when CDH was present. Vitamin E treatment did not improve either lung growth or function, although there was a trend toward less CDH. We have shown, for the first time, that the lung hypoplasia seen in nitrofen-exposed rat fetuses is associated with a dramatic reduction in static lung function, even when CDH is not present. Finally, our findings support the notion that lung hypoplasia in the nitrofen-rat model is independent of CDH formation. **(*Pediatr Res* 57: 392–395, 2005)**

Abbreviation

CDH, congenital diaphragmatic hernia

Congenital diaphragmatic hernia (CDH) is thought to result from failure of the pleuroperitoneal canal to close *in utero* between 8 and 10 wk of gestation (1). As a result, intra-abdominal contents herniate into the thoracic cavity, leading not only to hypoplastic development of the ipsilateral lung but also to abnormal development and function of the heart and the contralateral lung. CDH occurs in approximately one of every 4000 live births and carries substantial morbidity risks. Approximately 60% of infants with CDH will die in the immediate neonatal period, and survivors often have significant, life-long cardiopulmonary dysfunction. Because the defect and the associated maldevelopment occur early during fetal development, it is not surprising that early surgical repair and/or cardiopulmonary "rest" with extracorporeal bypass have not been successful in changing the dismal prognosis (2,3). Fetal surgery, although theoretically more promising, carries additional risks and even when technically successful has not been shown to affect outcome in infants with this condition (4,5).

A well-characterized animal model of CDH and pulmonary hypoplasia involves exposing the pregnant rat to 2,4-dichlorophenyl-p-nitrophenylether (nitrofen) before normal diaphragmatic closure (day 17 of gestation) (6,7). In this model, the timing of the fetal exposure has predictable consequences on both the incidence and the location (right *versus* left) of the diaphragmatic defect. For example, exposing the fetal rat to nitrofen at day 11 of development results in the majority of offspring having a right-sided CDH (7).

A variety of agents, including dexamethasone, epidermal growth factor, transforming growth factor β1, and vitamin A, have developmental and maturational effects on fetal animal lungs, and some, such as dexamethasone, have been shown to partially reverse the effects of nitrofen in animal models (8,9), although lung function was generally not assessed in these studies. This is important because mechanical interventions, such as tracheal ligation, have been shown to increase lung growth but do not improve function (10–13).

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The observation that antioxidants such as glutathione and vitamin C independently accelerate fetal lung growth has led investigators to examine the effects of other antioxidants, such as vitamin E. Although these investigators were able to show that vitamin E improved lung growth both *in vitro* and *in vivo*, lung function was not studied (14,15). In addition, vitamin E was given well beyond the teratogenic window of nitrofen exposure, and the positive effects on lung growth were modest. Finally, in one of two previous studies, nitrofen-exposed animals without CDH were excluded (14), and in a later study, although all nitrofen-exposed fetuses were included, those with and without CDH were not analyzed separately (15). Thus, the effect of vitamin E on hypoplastic lungs without CDH could not be discerned. In the present study, we examined the effects of vitamin E, given at the time of nitrofen exposure to maximize teratogenic inhibition, and compared fetuses with and without CDH to determine the independent effect of nitrofen on lung development. We also report the first study of the effect of vitamin E on lung function in this model.

METHODS

Animals. Timed-pregnant Sprague-Dawley rat dams (225–275 g) were gavage-fed nitrofen (320 mg/kg) on gestational day 11 (term is 22 d), after an initial fast. (Day 11 was selected to reduce the variability in left *versus* right-sided CDH.) On the same day, dams were also given a s.c. injection of either vitamin E (140 mg/kg; 1 mg = 1 IU) in vehicle (alcohol) or vehicle alone. Several pregnant rats received neither nitrofen nor vitamin E and served as controls. Rat dams were anesthetized with an overdose of pentobarbital on day 21, and the fetuses were removed by an abdominal incision; fetuses then were individually anesthetized with an overdose of pentobarbital. The study was approved by the Institutional Animal Care and Use Committee.

Agents. Purified Nitrofen (Cerilliant Corp., Round Rock, TX) was mixed with olive oil to a concentration of 100 mg/mL. A commercial preparation of vitamin E that contained primarily α-tocopherol (Sigma Chemical Co., St. Louis, MO) was diluted with ethanol to a concentration of 200 mg/mL.

Gross analysis. Rat pups were blotted dry and weighed. The abdomen then was opened by incision, and the presence of CDH was determined. The trachea then was ligated and cannulated with a 22-G catheter (Angiocath; Becton-Dickinson, Franklin Lakes, NJ), and static pressure volume curves were measured. Lungs then were removed, separated, weighed, homogenized, and refrigerated for later DNA and protein analyses.

Biochemical analysis. Lungs were initially frozen in liquid nitrogen and stored at −80°C until later homogenized in either 30 mL (for the normally smaller, left lung) or 40 mL (for the normally larger, right lung) of PBS. Homogenates then were analyzed for DNA and total protein content. DNA content was determined by a fluorometric method using bisbenzimidole (American Hoechst, Bridgewater, NJ) using commercially available bovine pancreatic DNA standards (Sigma Chemical Co.) (16). Fluorescence was read at Ex 356, Em 458 nm in a fluorocolorimeter (Molecular Devices, Sunnyvale, CA) at 37°C. Protein content was measured by the Bio-Rad method (Bio-Rad Laboratories, Richmond, CA) (17).

Pulmonary function. Cannulated pups were connected to a calibrated pressure transducer (Grass Instruments Co., Quincy, MA), precalibrated using a water manometer. Static pressure-volume curves were generated by a recently described method using the principle of Archimedes (18). Briefly, the pup was immersed in a saline-filled, 250-mL beaker sitting on an electronic balance, accurate to ±0.0001 g. A three-way stopcock allowed instillation of air into the fetal lungs using a 10-mL glass syringe. Each 1-μL change in volume displacement within the pup's lung resulted in a 1-mg change in recorded weight of the system. The lungs were pressurized in 5-cm H_2O increments, until a maximum pressure of 35 cm H_2O was reached, then deflated in a like manner. The static volume at the first step in deflation (30 cm H_2O) was taken as a measure of surface tension recoil pressure.

Statistical analysis. Data are reported as mean ± SD. Groups were compared using unpaired *t* tests for continuous variables and χ^2 analysis for discrete variables, using commercial software (Statview; Abacus Concepts, Berkeley, CA). A $p < 0.05$, corrected for multiple comparisons when appropriate, was taken as indicating a significant difference.

RESULTS

A total of 203 fetuses, born of 22 dams, were studied. Of 151 nitrofen-exposed fetuses, 77% had CDH; 92% of these were right-sided, and 5% were bilateral. The incidence of CDH was 82% in control fetuses and 71% in vitamin E–treated fetuses ($p = 0.17$).

Because the vast majority of CDH that occurred was right-sided, we excluded fetuses with left-sided CDH (*i.e.* either isolated or bilateral) from further analysis and compared measures of right lung growth and development. We found that nitrofen-exposed fetuses not only were smaller than nonexposed fetuses but also had disproportionately smaller lungs and poorer lung function, despite higher DNA content relative to lung weight (Table 1). These differences seemed to be due primarily to nitrofen exposure and not the presence of CDH (Table 2). However, the poorer lung function seen in nitrofen-exposed fetuses was even worse when CDH was present (Table 3).

Vitamin E treatment at the time of nitrofen exposure did not seem to improve either lung growth or function. In fetuses without CDH, vitamin E treatment was associated with significantly higher DNA/protein ratios, suggesting cellular hypoplasia (Table 2). In fetuses with CDH, vitamin E treatment was associated with higher total lung DNA but no differences in lung weight or function (Table 4).

DISCUSSION

In a nitrofen model of CDH, we found that exposure at day 11 of gestation in the pregnant rat leads to 72% of their fetuses having an isolated, right-sided CDH. Previous experience suggested that earlier exposure (*i.e.* gestational day 8–9) might result in a variable percentage of left- and right-sided CDH, whereas we found that later exposure ensured a more homo-

Table 1. *Comparison of control and nitrofen-exposed fetuses, with or without CDH*

				Right lung measurements			
	N	Body weight (g)	Total lung DNA (mg)	Weight/body weight (mg/g)	DNA/lung (mg/g)	DNA/protein (mg/g)	Deflation volume (μL/g body weight)
Control	52	5.27 ± 0.34	907 ± 195	16 ± 3	6.3 ± 1.5	233 ± 54	51 ± 7
Nitrofen	78	4.45 ± 0.31*	668 ± 200*	11 ± 4*	8.6 ± 3.3*	213 ± 77	25 ± 12*
Nit + Vit E	64	4.35 ± 0.47*	772 ± 196*†	12 ± 5*	9.9 ± 4.7*†	281 ± 83*‡	27 ± 14*

* Significantly different from control, $p < 0.0005$, by ANOVA.
† Significantly different from nitrofen alone, $p < 0.05$, by ANOVA.
‡ Significantly different from nitrofen alone, $p < 0.0001$, by ANOVA.

Table 2. *Comparison of control and nitrofen-exposed fetuses, without CDH*

				Right lung measurements			
	N	Body weight (g)	Total lung DNA (mg)	Weight/body weight (mg/g)	DNA/lung (mg/g)	DNA/protein (mg/g)	Deflation volume (μL/g body weight)
Control	52	5.27 ± 0.34	906 ± 195	16 ± 3	6.3 ± 1.5	233 ± 54	51 ± 7
Nitrofen	15	4.54 ± 0.40*	726 ± 262*	12 ± 3*	7.9 ± 2.7*	221 ± 95	35 ± 17*
Nit + Vit E	19	4.31 ± 0.58*	816 ± 163	13 ± 3*	8.7 ± 1.9*	306 ± 80*†	37 ± 18*

* Significantly different from control, $p < 0.005$, by ANOVA.
† Significantly different from nitrofen alone, $p < 0.0001$, by ANOVA.

Table 3. *Comparison of nitrofen-exposed fetuses with (right only) and without CDH*

			Right lung measurements			
	N	Body weight (g)	Weight/body weight (mg/g)	DNA/lung (mg/g)	DNA/protein (mg/g)	Deflation volume (μL/g body weight)
Right CDH	108	4.40 ± 0.34	11 ± 5	9.5 ± 4.4	236 ± 82	23 ± 9*
No CDH	34	4.41 ± 0.52	13 ± 3	8.3 ± 2.3	269 ± 96	36 ± 17

* Significantly different from no CDH, $p < 0.0001$, by ANOVA.

Table 4. *Comparison of nitrofen-exposed fetuses with right-sided CDH*

				Right lung measurements			
	N	Body weight (g)	Total lung DNA (mg)	Weight/body weight (mg/g)	DNA/lung (mg/g)	DNA/protein (mg/g)	Deflation volume (μL/g body weight)
Nitrofen	63	4.43 ± 0.29	654 ± 182	11 ± 4	8.8 ± 3.5	212 ± 72	22 ± 8
Nit + Vit E	45	4.37 ± 0.41	753 ± 208*	12 ± 6	10.4 ± 5.3	270 ± 82	23 ± 9

* Significantly different from nitrofen alone, $p < 0.01$, by ANOVA.

geneous population of almost exclusively right-sided CDH. Similar to previous reports, we found that nitrofen exposure was associated with generalized poor growth, as measured by total body weight, and disproportionately small lungs with evidence of cellular hypoplasia. For the first time, we also showed that nitrofen exposure led to poor pulmonary mechanical function. We did not find that vitamin E treatment at the time of nitrofen exposure significantly ameliorated any of these effects.

The nitrofen-exposed rat model has been used often to study CDH, but the effect of nitrofen in fetuses without CDH has not been well studied. This is important, because some of the abnormal lung findings in previous studies of CDH in this model may have been due to nitrofen exposure *per se* and not the associated CDH. Our data confirm previous observations that nitrofen can induce pulmonary hypoplasia independent of CDH. This has been previously suggested by others (19,20), although the concept of lung hypoplasia occurring before development of CDH has been disputed by others. The findings in our study, along with the recent observation that nitrofen-exposed fetal lungs show evidence of hypoplasia before the development of CDH (21), clearly support the concept that lung hypoplasia in CDH is not a secondary phenomenon. It is possible that the primary defect in nitrofen-induced CDH is lung hypoplasia and that the diaphragmatic defect may actually be a secondary phenomenon, and some mechanisms by which this might occur have been discussed (22). However, a recent series of studies in mutant mice suggests that the diaphragmatic defect in the nitrofen model is independent of myogenesis and lung formation (23).

We have shown for the first time that the lung hypoplasia seen in the nitrofen model of CDH is associated with a dramatic reduction in static lung function. Whether this was solely due to the obvious lung hypoplasia or there was also a functional surfactant deficiency could not be discerned by our methods. Although surfactant dysfunction has been shown in human infants with CDH (24) and in some animal models of CDH (25,26), a recent study in the nitrofen-rat model found that surfactant protein expression was not altered in rats with nitrofen-induced CDH (27). However, in all of these animal studies, only fetuses with CDH were examined. We found evidence of severe pulmonary hypoplasia in nitrofen-exposed fetuses regardless of CDH, although static lung volume was significantly worse when CDH was present. We have also shown that nitrofen exposure leads to generalized poor fetal growth, with disproportionately poor lung growth and development. This adds further support to previous observations that the nitrofen model shows a striking similarity to the human phenotype.

Two previous studies (14,15) concluded that vitamin E had a protective effect on lung growth and development in the nitrofen model of CDH. In those studies, vitamin E was given to pregnant rats daily for 5 d, beginning 1 wk after exposure to nitrofen. The authors found that treatment with vitamin E was associated with increased lung weights and total lung DNA but no change in lung DNA concentration or DNA:protein ratios suggesting accelerated growth of the hypoplastic lungs. We also found that vitamin E treatment resulted in significantly higher total lung DNA, but nitrofen-exposed fetuses still had significantly less total lung DNA than control fetuses. In one of the previous studies, a control group was not included, so the magnitude of the effect of vitamin E supplementation could not be discerned (14). In a more recent study, a control group was included and the effect of vitamin E supplementation was

actually modest; indeed, in that study, vitamin E–treated, nitrofen-exposed fetuses had significantly lower lung weights, lung DNA, and lung protein than the control fetuses (15). Similar to this study, we were able to show that the improved lung growth seen with vitamin E treatment was actually modest, and these nitrofen-exposed fetuses still had significantly hypoplastic lungs compared with normal fetuses. This was further evident when we examined mechanical function; vitamin E–treated fetuses had static lung volumes no different from control fetuses. It is intriguing to note that there was a trend toward less CDH in vitamin E–treated fetuses, although this, too, was a modest effect.

There are at least two methodologic differences between our study and the two previous studies noted above. First, the total dose of vitamin E used by us was somewhat lower. We gave a single depot injection of vitamin E, whereas the two previous studies noted above gave several daily doses of vitamin E intragastrically. Second, the timing of vitamin E supplementation was different. We gave vitamin E at the time of nitrofen exposure, hypothesizing that if it worked by blocking the teratogenic effects of nitrofen, then the modest effects seen in other studies may have been because vitamin E was not given until several days after nitrofen exposure. However, our data refute this hypothesis.

Although it is possible that a higher does of vitamin E may have shown more positive effects, this seems unlikely for several reasons. First, the dose of vitamin E that we used is already large, when one considers that a dose of 3.5 mg/d is sufficient to maintain normal vitamin E levels in premature neonates who weigh $<$1000 g (28); we gave a dose that was 10-fold higher than this to pregnant rats that typically weighed 250 g. Second, although the two previous studies noted above gave 4–20 times as much vitamin E, they still found that vitamin E–treated fetuses were no bigger than control fetuses, a result that we found as well (14,15). Third, we administered the vitamin E on the same day as the nitrofen, ensuring that the highest concentration of vitamin E was present during the time when nitrofen should have been inducing its effects.

CONCLUSION

In conclusion, nitrofen not only is a potent inducer of CDH in the rat model but also results in significant lung hypoplasia in fetuses without CDH. These effects are only modestly ameliorated by concurrent treatment with vitamin E. Our data add further to the concept that in the nitrofen model of CDH, which shares much homology with the human phenotype, lung hypoplasia may be an entirely separate phenomenon from CDH formation. This notion has received strong support from the elegant work of Babiuk *et al.* (23) showing that diaphragmatic defects can occur independent of lung formation. This idea also agrees well with the clinical observations that fetal diaphragmatic repair has not improved survival in cases of CDH (4) and that lung hypoplasia seen with CDH is not limited to the ipsilateral lung (1). Further advances in this area will need to uncover the actual mechanism(s) by which lung hypoplasia develops in this disorder.

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