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Prenatal Glucocorticoid Therapy Reverses Pulmonary Immaturity in Congenital Diaphragmatic Hernia in Fetal Sheep

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Objective

To assess the feasibility of conducting clinical trials of prenatal steroid therapy for congenital diaphragmatic hernia (CDH) in humans, the authors tested whether prenatal glucocorticoid, currently the standard treatment to minimize respiratory distress syndrome in premature infants, might improve the pulmonary immaturity in severe CDH in a large animal model.

Summary Background Data

The authors have used the nitrofen-induced rat model of CDH, which demonstrates immature lungs by biochemical, morphometric, and molecular biologic criteria. They also have shown that the lethally immature lungs of the full-term CDH rats can be improved by biochemical, morphometric, physiologic, and molecular criteria by treating the mothers with parenteral steroids at doses extrapolated from the current therapy used to accelerate lung development of premature human babies.

Methods

During a 3-year period in 88 fetal sheep, 1) left-sided diaphragmatic hernias were created surgically at varying gestational ages (day 78–90; term = 142–145 days) and size to maximize severity (n = 45), 2) placement and design of indwelling fetal intravenous catheters were optimized (n = 13), and 3) timing and dosage of cortisol administration were determined (n = 17). As a result, diaphragmatic hernias were created on day 80, intravenous catheters were placed on day 120, and twice-daily intravenous cortisol injections (n = 8) or saline as the control (n = 5) were administered (days 133–135). Lambs were delivered on day 136 via cesarean section to avoid steroid-induced abortion; vascular access was obtained, and the fetuses were ventilated at standard settings. Physiologic data were collected, and lungs were harvested for biochemical and histologic analysis.

Results

Significant improvements were measured in postductal arterial oxygen pressure ([PaO_2] 38 ± 6 mmHg after cortisol therapy compared with 20 ± 3 mmHg for saline controls; p = 0.002) and in dynamic compliance (0.42 ± 0.05 mL/cm H_2O *vs.* 0.29 ± 0.01 mL/cm H_2O; p = 0.01). Lung glycogen levels in the right lung of the cortisol group were significantly better than controls (4.6 ±

0.3 mg/g lung *vs.* 6.8 ± 0.4 mg/g; p = 0.002), as were protein/DNA levels (8.3 ± 0.9 mg/mg *vs.* 14.5 ± 2.9 mg/mg; p < 0.05). Striking morphologic maturation of airway architecture was observed in the treated lungs.

Conclusions

Prenatal glucocorticoids correct the pulmonary immaturity of fetal sheep with CDH by physiologic, biochemical, and histologic criteria. These data, combined with previous small animal studies, have prompted the authors to initiate a prospective phase I/II clinical trial to examine the efficacy of prenatal glucocorticoids to improve the maturation of hypoplastic lungs associated with CDH.

Despite intensive investigation and aggressive therapeutic intervention, newborn infants with immediate postpartum respiratory distress secondary to an underlying congenital diaphragmatic hernia (CDH) continue to have unacceptably high morbidity and mortality.[1] Pulmonary hypoplasia and persistent pulmonary hypertension frustrate efforts to maintain gas exchange with conventional ventilation. Newer therapies to support these infants, including high-frequency ventilation, extracorporeal membrane oxygenation, surfactant therapy, nitric oxide, and prenatal surgery, have made inroads into improving survival, but more progress is required to make the outcomes acceptable.

The lungs of babies dying from CDH resemble the immature lungs of the premature newborn with respiratory distress syndrome, as evidenced by surfactant deficiency, impaired pulmonary compliance, and the formation of hyaline membranes.[2-7] Similarly, we observed that the lungs of rats with nitrofen-induced CDH were biochemically and histologically immature.[8] Prenatal glucocorticoids given to infants at risk for premature delivery at early canalicular stages of development, similar to those seen with CDH lungs, successfully improved premature lungs in animals and humans.[9-11] Therefore, we hypothesized that prenatal glucocorticoid, currently the standard treatment to prevent hyaline membrane disease in premature humans,[9,12] also might correct the parameters of pulmonary biochemical and morphologic immaturity of CDH. We tested this hypothesis in a series of rodent experiments; prenatal dexamethasone improved the known parameters of lung immaturity and hypoplasia.[13-17] Specifically, dexamethasone treatment increased the lung disaturated phosphatidylcholine content, reduced the lung glycogen concentration, reduced the saccular septal thickness, and increased the mean saccular size and volume fraction of saccules in the lungs of rats with large congenital diaphragmatic hernia, in comparison to CDH rats not treated.

To test functional improvement, we developed a technique based on Archimedes' principle to determine accurately *in situ* fetal rat lung static compliance as a physiologic correlate of biochemical, morphometric, and molecular improvement.[14] Functional residual capacity normalized and compliance improved in CDH lungs to the point at which they were indistinguishable from normal non-CDH lungs.[15] We also applied quantitative stereologic morphometric techniques to evaluate pulmonary development after prenatal hormone therapy in CDH rats.[16] Morphologically, the CDH lungs from the dexamethasone-treated group revealed striking maturational changes compared with the lungs of normal saline-treated controls, which were arrested developmentally at the canalicular stage.[15,16] Finally, using Northern analysis, we demonstrated significant induction of surfactant associated proteins A and B mRNA levels at day 18 of gestation after prenatal glucocorticoid treatment from days 16 to 17.[17]

Thus, prenatal glucocorticoid treatment improved parameters of profound pulmonary hypoplasia including glycogen, disaturated phosphatidylcholine, pulmonary compliance, morphometrics, and surfactant-associated proteins A and B mRNA levels in a rodent model of pharmacologically induced CDH. Based on these data, we carried these experiments forward to a large animal model of surgically induced CDH to eliminate any unknown genetic effects from the nitrofen and to prove functional postnatal benefit, as a stepping-stone to clinical trials.

MATERIALS AND METHODS

All protocols were approved by the Massachusetts General Hospital Subcommittee on Research Animal Care and conform to National Institutes of Health guidelines. We began by creating CDH surgically in sheep (n = 45) at varying gestational ages (day 78–90) to select the

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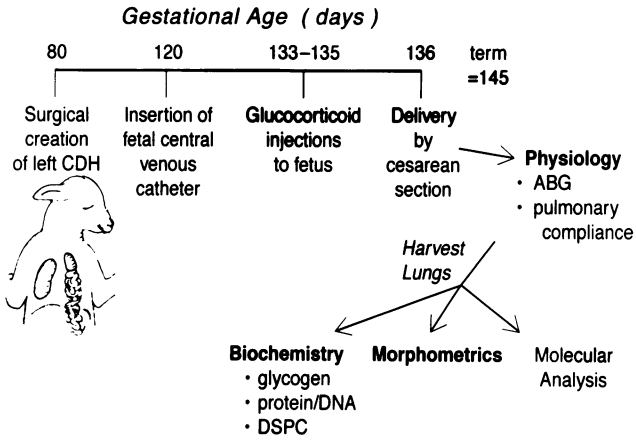

Figure 1. Schematic diagram of selected experimental design.

most severe pulmonary lesion consistent with survival to term (days 142–145) after fetal intravenous catheter placement (day 120) and administration of glucocorticoids. Creation on day 80 resulted in severe pulmonary hypoplasia with acceptable rates of survival to term. Glucocorticoids do not cross the placenta in sheep as they do in rats and humans[10,18,19]; therefore, at 120 days gestation, it was necessary to insert an indwelling silastic catheter.[10] We obtained long-term fetal intravenous access (n = 13) by creating a hybrid catheter consisting of a subcutaneously implanted Portacath (Davol, Inc., Cranston, RI) spliced to a Broviac catheter (Bard Access Systems, Salt Lake City, UT). The catheter was tunneled from its subcutaneous position in the flank of the pregnant ewe into the ewe's peritoneum, through the uterine wall, where it was secured by a purse string, and then into the right neck of the fetus. The cuff was secured under the fetus' skin, and the catheter was advanced via a venotomy in the right jugular vein of the fetus into a central position.

We based our cortisol dosage and administration schedule on extrapolations from treatment regimens used routinely in humans undergoing preterm labor.[12] Initially, four doses of cortisol (33 mg each) were administered intravenously to the fetus every 12 hours on days 135 and 136 (low-dose cortisol CDH, n = 17). Physiologic and biochemical measurements were performed (description follows), and no improvements were observed with this lower-dose glucocorticoid therapy, with the exception of protein/DNA levels. We next increased each dose to 50 mg, and attempted to continue twice-daily injections for 5 days. However, all of these fetuses aborted spontaneously early because of the labor-inducing effect of glucocorticoids in sheep (but not humans).

Based on these experiments, we selected the ultimate experimental design, illustrated in Figure 1. Diaphragmatic hernias were created surgically in fetal sheep by techniques previously developed[20–23] at 80 days gestation. Pregnant ewes with ultrasonographic evidence of twins were preferentially selected; both fetuses had a CDH created, but only one fetus from each pair received steroid therapy. Singleton fetuses were used to complete the groups.

Beginning at 133 days gestational age, one fetus of each pair received twice-daily intravenous injections of cortisol (hydrocortisone sodium succinate, 125 mg/mL, Abbott Laboratories, North Chicago, IL) 50 mg/dose every 12 hours for 3 days (6 doses, cortisol CDH, n = 8), whereas the paired twin received an equal volume of normal saline (saline CDH controls, n = 5). There was no danger of the control twin receiving steroids inadvertently from metabolic products because the fetuses had separate amniotic sacs. This dose schedule was extrapolated from the human doses used effectively for prematurity and is similar to previously reported doses used in non-CDH fetal sheep.[10,19]

Fetuses were delivered via cesarean section at 136 days gestation to minimize the risk of intervening labor (term = 142–145 days). Fetal respiration was prevented by covering the head of the anesthetized lamb with a rubber glove. The fetus was maintained on placental circulation under anesthesia during subsequent manipulations.[23,24] Intravenous and intra-arterial catheters were placed by open femoral cannulation, and tracheotomies were performed with a 3.5-mm endotracheal tube. After clamping and dividing the umbilical cord, the fetuses were weighed and then ventilated at standard settings (peak inspiratory pressure/positive end-expiratory pressure 30/3 cm H_2O, respiratory rate [RR] 30 breaths/minute, fraction of inspired oxygen 100%) for 30 minutes.

Heart rate, arterial blood pressure via transduced arterial catheter, and respiratory rate were measured continuously and recorded every 5 minutes and whenever there was a substantial change. An in-line dynamic pulmonary function monitor was connected to the endotracheal tube, and volume-pressure loops were collected from which parameters of pulmonary compliance were calculated (dV/dP). Postductal arterial blood gas determinations (arterial oxygen pressure [PaO_2], partial arterial pressure of carbon dioxide [$PaCO_2$], and pH) were taken every 5 minutes.

For each animal, after completion of the physiologic measurements, the right upper, right middle, and left upper lung lobes were harvested, rinsed in saline, flash-frozen in liquid nitrogen, and stored at −80 C for biochemical analyses. Thawed samples were weighed and then assayed, as described previously, for glycogen content, disaturated phosphatidylcholine, DNA, and total protein.[8] The lower lobes were distended with 10% formalin, fixed, embedded in paraffin, sectioned, and stained for

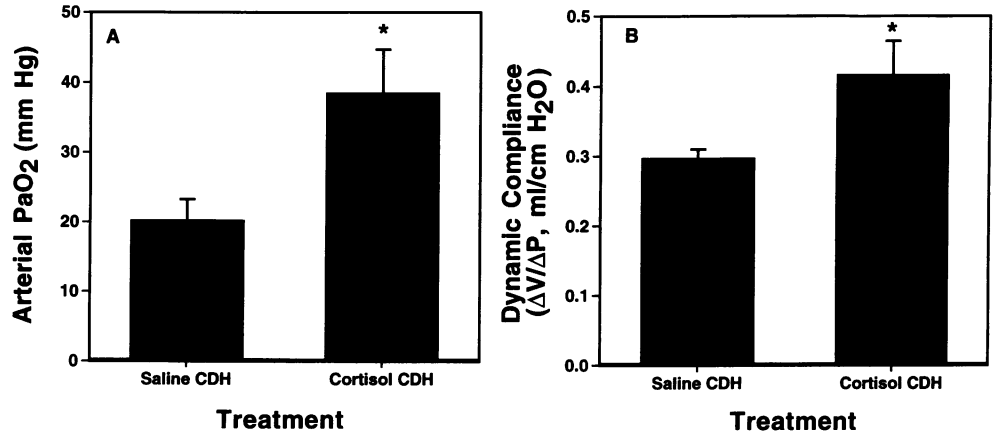

Figure 2. Physiological measurements in fetal sheep with congenital diaphragmatic hernia (CDH) after prenatal glucocorticoid therapy. (A) Postductal arterial oxygen pressure (PaO_2) measurements (mmHg) for high-dose cortisol therapy (cortisol CDH) *vs.* saline controls (saline CDH) showed significant improvement (mean ± standard error of the mean; *p = 0.002). (B) Corresponding improvements were measured in dynamic compliance after prenatal steroid therapy; *p = 0.01.

histology. Lungs from normal non-CDH age-matched fetuses were used for comparisons (non-CDH, n = 7).

All data are presented as mean ± standard error of the mean. Statistical comparisons were made using Student's t test or analysis of variance with the Bonferroni-Dunn test.[25]

RESULTS

Physiologic measurements showed a significant improvement in postductal PaO_2 (Fig. 2A) with high-dose cortisol treatment from 20 ± 3 mmHg to 38 ± 6 mmHg (mean ± standard error of the mean, p = 0.002 compared with normal saline CDH controls by Student's t test). Similar improvements were observed in dynamic compliance from 0.29 ± 0.01 mL/cm H_2O to 0.42 ± 0.05 mL/H_2O (Fig. 2B, p = 0.01 for high-dose cortisol *vs.* normal saline CDH controls). The lower-dose cortisol regimen resulted in no significant changes in either parameter.

Significant salutary changes were seen in lung glycogen levels for the right lung after high-dose cortisol therapy (Fig. 3). Lung glycogen levels normally decrease near term, so that a decreased glycogen level is an indication of pulmonary maturation. Lung glycogen levels in the right lung of the high-dose group were significantly lower (4.6 ± 0.3 mg/g lung) than the saline CDH control group (6.8 ± 0.4 mg/g, p = 0.002 by Bonferroni-Dunn test and analysis of variance). This improvement reached a level very similar to that on non-CDH control right lung (4.43 ± 0.24 mg/g lung). Although there was a trend in the severely hypoplastic left lung, the decrease in glycogen failed to achieve statistical significance. For each CDH group, the left lung glycogen level was significantly higher than the corresponding right lung level, demonstrating greater biochemical immaturity on the left than the right in this model of CDH.

Protein/DNA levels (mg/mg) were higher in normal saline CDH lungs than in normal lungs (p = 0.0001), and

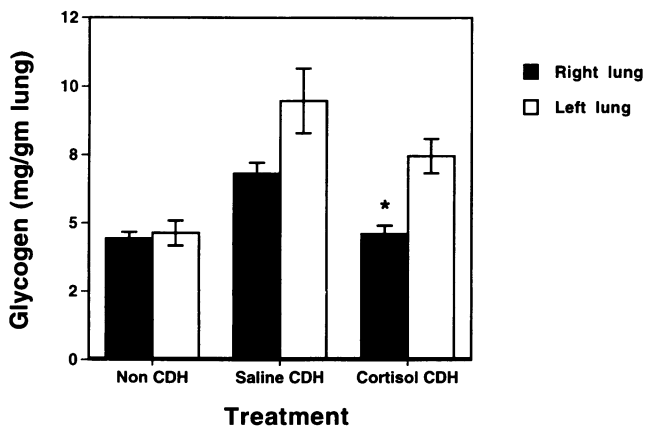

Figure 3. Lung glycogen levels (mg/g lung) for three treatment groups segregated by right and left lungs, demonstrating improvement in right lung glycogen levels after glucocorticoid therapy to near normal levels; *p = 0.002.

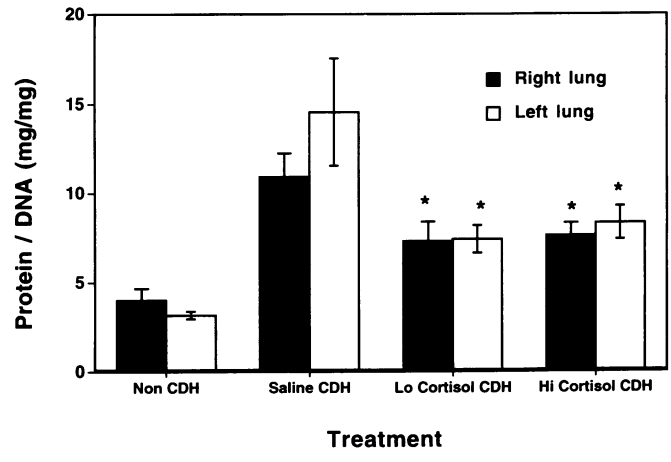

Figure 4. Protein/DNA levels (mg/mg) improved after both dosage schedules of prenatal cortisol (lo cortisol CDH = low-dose cortisol regimen, hi cortisol CDH = high-dose cortisol regimen; *p < 0.03 compared with saline CDH; CDH = congenital diaphragmatic hernia).

Table 1. DISATURATED PHOSPHATIDYLCHOLINE/DNA CONTENT OF CONGENITAL DIAPHRAGMATIC HERNIA (CDH)

	Treatment			
	Saline CDH	Low-Dose Cortisol CDH	High-Dose Cortisol CDH	Non-CDH
Right lung	0.54 ± 0.05	0.58 ± 0.08	0.54 ± 0.03	0.61 ± 0.04
Left lung	0.52 ± 0.05	0.56 ± 0.05	0.51 ± 0.05	0.55 ± 0.03

were significantly lower than normal saline CDH lungs after both low- and high-dose prenatal cortisol therapy (cortisol CDH) for both left and right lungs (Fig. 4, $p < 0.05$). Disaturated phosphatidylcholine/DNA levels were unchanged by treatment and were the same for right and left lungs within treatment groups (Table 1). No differences were observed in heart rate, blood pressure, or $PaCO_2$ among the groups (data not shown). We observed striking qualitative improvements in morphology in fetal sheep CDH lungs after prenatal high-dose cortisol therapy (Fig. 5). The treated lungs demonstrated thinning of the interstitium, more mature alveoli with thinner walls, and improved aeration, compared with age-matched controls.

DISCUSSION

Prenatal glucocorticoids can improve parameters of immaturity in surgically created CDH lungs in sheep, as previously shown in the lungs of rodents with nitrofen-induced CDH.

The incidence of congenital diaphragmatic hernia in the human population is more common than previously appreciated, occurring in approximately 1 in 2000 pregnancies[26] and 1 in 2400 to 3000 live births,[1,27]; thus, in the United States, with 4 million live births, this leads to nearly 1300 cases per year. The 60% mortality rate of CDH[1,28] would result in approximately 800 deaths a year, which is 25% of deaths due to congenital heart disease in the first year of life, or 1.3 times the number of deaths due to leukemia from age 0 to 14 years.[29,30]

This mortality persists despite numerous advances in neonatal intensive care, including nitric oxide, surfactant replacement, high-frequency ventilation, extracorporeal membrane oxygenation, and delayed surgery for CDH, which incorporates the use of prosthetic material if necessary.[31] Those surviving extracorporeal membrane oxygenation have a high morbidity of developmental delay, seizure activity, bronchopulmonary dysplasia, gastroesophageal reflux, and deafness.[32] Consequently, those that survive may require a high level of prolonged, sophisticated, costly care. In fact, the per-patient, in-hospital cost for a CDH infant surviving extracorporeal membrane oxygenation has been found to be $365,000.[33] Therefore, our aim is to treat these infants *in utero* to increase lung development before birth, and thereby to improve the response to current therapies, while averting high morbidity and mortality, and coincidentally lowering the economic burden imposed by the defect.

In developing the optimal experimental protocol (Fig. 1), two important issues had to be addressed in sheep, in which—unlike humans and rats—glucocorticoids do not cross the placenta and induce preterm labor and delivery.[34,35] Therefore, we designed and constructed a hybrid catheter to deliver intravenous glucocorticoid therapy to the fetus *in utero,* then tested two other cortisol administration doses and schedules, one of which induced preterm labor; the other failed to achieve satisfactory pulmonary maturation before adopting the scheme employed.

Prenatal glucocorticoid therapy improved postductal PaO_2 (Fig. 2) and dynamic compliance in sheep treated intravenously on days 133 to 135 and delivered on day 136. Because postductal PaO_2 has been shown to be a very strong predictor of survival in humans with CDH,[36] the observed significant improvement in PaO_2 in the lambs after prenatal glucocorticoid therapy may similarly be predictive of benefit from this treatment in humans.

Prenatal glucocorticoid therapy induced significant maturation of right lung, as reflected by glycogen levels (Fig. 3), which were already better than the more immature left lung in animals with CDH. These differences recapitulate the size and functional asymmetries that have been described previously in humans.[37] The observed reduction in protein/DNA after prenatal glucocorticoid therapy (Fig. 4) is consistent with previous ontogeny studies[38] and suggests that the more mature pulmonary architecture contains cells of decreased volume, as would be expected for efficient gas exchange, and as was observed histologically (Fig. 5).

Before embarking on a clinical trial, the safety of the mother and fetus with prenatal glucocorticoid administration must be considered. Fortunately, there is a large accumulated clinical experience with this therapy for preterm labor and delivery. Potential complications from prenatal corticosteroid treatment of pregnant women at risk for premature delivery recently was summarized (1994) at the National Institutes of Health Consensus Development Conference on the Effect of Corticosteroids for Fetal Maturation on Perinatal Outcomes[39] and included published meta-analyses.[40] Data from five major studies, representing

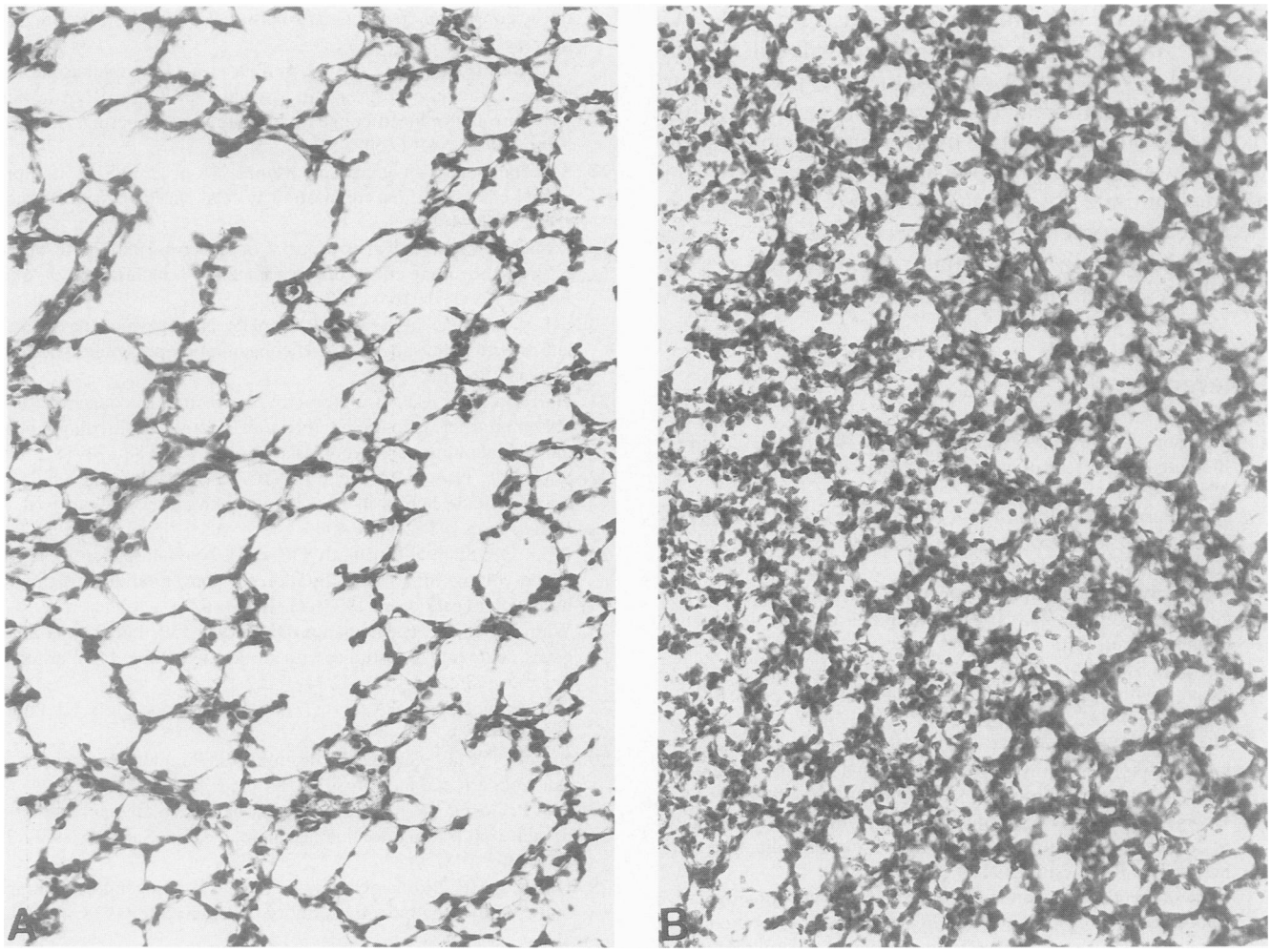

Figure 5. Light micrographs of congenital diaphragmatic hernia lungs after prenatal cortisol therapy (A) *vs.* saline controls (B), demonstrating maturation (hematoxylin and eosin staining, original magnification 200×).

more than 35,000 infants, indicated no serious adverse effects for the mothers or infants. Documented benefits included reduced risk of mortality, reduced intraventricular hemorrhage, respiratory distress syndrome, and air leak; because the small increase in the risk of necrotizing enterocolitis ([NEC] 7.1% incidence, odds ratio 1.29) observed could be attributed to the prematurity of the infants in the study, the expected risk to term or near-term infants with CDH may be lower. Two longitudinal studies have documented the absence of demonstrable long-term adverse effects in subsequent growth, development, or behavioral outcomes.[41,42] Prenatal glucocorticoid administration does not suppress the pituitary-adrenocortical response postnatally.[43] Furthermore, the available data strongly suggest no increased adverse effects from infectious complications.[39] The glucocorticoids, dosages, and schedules of administration used in this study were comparable to those proposed for the *in utero* treatment of humans with CDH.

Based on our promising results, previously in the rodent, and in the sheep CDH model, and the lack of adverse outcomes after extensive prenatal use for respiratory distress syndrome, we are initiating a two-center, randomized, double-blind, placebo-controlled phase I/II human clinical trial of prenatal glucocorticoid therapy for CDH. We hypothesize that a strategy of prenatal pharmacologic intervention to accelerate lung development and maturation will improve the dismal survival in newborn infants with CDH-associated pulmonary hypoplasia.

This experimentally based therapy has appeal because it is noninvasive and is based on pharmacology with which both obstetricians and pediatricians are comfortable. Clinically, this approach can be used in conjunction with all other efficacious approaches: conventional, experimental, prenatal, and postnatal. *In utero* surgical repair of the defective diaphragm[44] and pulmonary distension by tracheal occlusion or fluid distension of the airway[45] are exciting avenues that potentially could be

used synergistically with prenatal glucocorticoids. We would expect that the sickest infants will still require maximal neonatal intensive care therapy, postnatal surfactant, extracorporeal membrane oxygenation, nitric oxide, and other supportive measures to which we anticipate they will be more responsive. It also may be reasonable to continue glucocorticoids into the postnatal period because some benefit has been obtained with this therapy postnatally for premature infants with respiratory distress syndrome.[46,47]

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Discussion

DR. W. HARDY HENDREN (Boston, Massachusetts): The severely ill newborn with congenital diaphragmatic hernia continues to be one of the unsolved problems in pediatric surgery.

Dr. Schnitzer and his coworkers are to be congratulated for showing in this very important and elegant study that prenatal administration of steroids can improve the maturation and function of the hypoplastic lung, which continues to kill these babies despite all the changes that Dr. Schnitzer has just shown in our clinical management that have taken place in the last decade.

I would like to ask, Dr. Schnitzer, if you think that there is any down side to this therapy judging from its prior use to prevent premature delivery in threatened abortion or in those infants who have been treated with steroids for the respiratory distress syndrome. Do you think it is possible that growth, in terms of cell number and size of the lung, and maturation may be mutually exclusive?

Dr. Jay Wilson in our department at Children's Hospital in Boston has shown impressive growth of the hypoplastic lung in sheep after birth by ventilating it with perfluorocarbon solution, which is inert and not absorbed.

A limited clinical trial has just been completed using this technique in collaboration with Dr. Ronald Hirschl in Dr. Arnold Coran's department in Ann Arbor, Michigan, with very encouraging results. A ten-center clinical trial has been approved by the Food and Drug Administration, and will soon begin.

I wonder if you could comment, Dr. Schnitzer, on the possible use of antenatal steroids in concert with postnatal perfusion of the hypoplastic lung in these babies. Again, congratulations for providing us with another piece of this difficult clinical puzzle.

DR. JAY L. GROSFELD (Indianapolis, Indiana): The authors are to be congratulated for the fine quality of their work and their perseverance for carrying this experiment out in a variety of animal models for more than 3 years.

They were clever to observe that dexamethasone administered to mothers at risk for delivering premature babies improved lung maturation and survival and are now applying this concept to a congenital diaphragmatic hernia (CDH) model. Most babies with CDH are full-term babies. Although they are big babies, the ipsilateral lung is compressed *in utero*, is hypoplastic and immature, and just does not grow.

This is a very well-run experiment with superb controls. The control animal is a twin. So you have an experimental animal and a fetal control in the same mother.

The authors have clearly shown that steroid administration changes the lung morphometrics, biochemistry, and the partial pressure of oxygen (pO_2) levels.

I have just a couple of questions: We know that arterial partial pressure of carbon dioxide ($ApCO_2$) probably is the best indicator of alveolar ventilation. I did not observe any pCO_2 values either in the presentation or in the article. The $ApCO_2$ is an important clinical prognostic indicator in babies with CDH. Do you have any information regarding the effect of glucocorticoids on $ApCO_2$?

Second, we also know that you have absolutely superb pulmonary pathologists at your institution. I wonder whether they have actually documented and counted the number of type II pneumonocytes in your sheep fetuses after being treated with glucocorticoids? It is the type II pneumonocyte that defines lung maturation, because surfactant production from the type II pneumonocyte actually allows the alveoli to remain expanded after the onset of ventilation.

Finally, I wonder if you would be willing to speculate whether you believe pharmacologic maturation of the hypoplastic fetal lung will put to rest the need for fetal surgery to correct CDH?

I enjoyed this presentation very much and appreciated the authors making the manuscript available to me before the meeting.

DR. DAVID TAPPER (Seattle, Washington): I think that we are privileged to see studies in evolution. I think that Dr. Schnitzer clearly showed that they have taken this model from the small animal and moved it to sheep in preparation for evaluation in patients.

As Dr. Hendren pointed out, diaphragmatic hernias still have a significantly high mortality. Steroids given prenatally may increase lung maturation. These studies show that they definitely improve the physiologic parameters, improve the biochemical milieu, and alter the anatomy, so it makes intuitive sense for the authors to begin a trial in humans. I have just a few questions that I would like to ask them.