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Kidney development in the nitrofen-induced pulmonary hypolasia and congenital diaphragmatic hernia in rats☆

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Abstract

Background/Purpose: The relationship of the developing lung and kidney is not completely understood. Renal enlargement has been reported in association with pulmonary hypoplasia in congenital diaphragmatic hernia (CDH). Recent studies suggest that retinoids may be involved in the pathogenesis of CDH. The aims of this study were to investigate the effects of pulmonary hypoplasia on renal development and to evaluate retinoids status of kidneys in the nitrofen model of CDH.

Methods: Pregnant rats were exposed to either olive oil or 100 mg of nitrofen on day 9.5 of gestation. Fetuses were recovered at term and divided into 3 groups: 1, control (n = 69); 2, nitrofen without CDH (n = 25); and 3, nitrofen with CDH (n = 40). Kidneys were dissected, weighed, and processed for biochemical measurements of DNA, proteins, total retinol content, and for immunohistochemical staining of proliferating cells.

Results: Kidneys were smaller in nitrofen-exposed animals vs control animals (group 3, 0.65 ± 0.08; group 2, 0.62 ± 0.09 vs group 1, 0.73 ± 0.09% of body weight, $P < .001$), and there were no differences between right and left kidney weight in all the 3 groups. Regression of total kidney weight on body weight showed a linear direct correlation between them in all the groups. Total amount of DNA was significantly reduced in nitrofen-exposed animals vs controls (group 3, 80.58 ± 35.65; group 2, 64.71 ± 20.28 vs group 1, 110.34 ± 42.15 μg, $P < .01$), but the DNA concentration remained the same in the 3 groups (group 3, 3.59 ± 1.26; group 2, 3.06 ± 1.19; group 1, 3.43 ± 1.05 μg DNA/mg kidney). Total protein content (group 3, 1145.59 ± 500.36; group 2, 993.2 ± 276.62; group 1, 1287.48 ± 312.52 μg), protein concentration (group 3, 49.76 ± 11.12; group 2, 43.95 ± 6.79; group 1, 47.38 ± 6.93 μg protein/mg kidney), and protein-to-DNA ratio (group 3, 15.12 ± 5.98; group 2, 16.22 ± 6.85; group I, 16.16 ± 7.02 μg/μg) were similar in all groups. Retinol concentration was significantly reduced in both nitrofen-exposed groups compared with the control group (group 3, 1.35 ± 0.24; group 2, 1.28 ± 0.11; group 1, 2.53±0.61 μg retinol/g kidney). Proliferation index was similar in all 3 groups (group 3, 50.43 ± 8.81; group 2, 47.96 ± 6.01; group 1, 47.64 ± 5.76% of proliferating cells).

Conclusions: Our data clearly show that renal enlargement in association with pulmonary hypoplasia is not seen in the nitrofen-induced CDH. These results rule out any possible relationship between lung and kidney development. Moreover, kidneys are hypoplastic in both nitrofen-exposed groups and have

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reduced retinol content, suggesting that a retinoid pathway disruption could be the common mechanism in the pathogenesis of lung and kidney hypoplasia in the nitrofen model of CDH.

Pulmonary hypoplasia remains the main cause of high mortality in congenital diaphragmatic hernia (CDH) [1]. More than 10 years ago, Glick et al [2] presented the hypothesis that fetal pulmonary development is regulated by a pulmonary growth factor. According to their hypothesis the kidneys produce pulmonary growth factor resulting in a feedback signal from the lungs to the kidneys, a pulmonary-derived renotropin. They hypothesized that if lung growth was inhibited and pulmonary hypoplasia developed, this substance would be continuously released, resulting in kidney hyperplasia and secondary polyhydramnios, which is commonly seen in patients with CDH. Through autopsy reviews and surgically created animal models of CDH, these investigators demonstrated that kidneys were significantly larger than those of controls [2,3]. These findings were later challenged by other investigators who not only did not find the kidneys to be enlarged, but to be significantly smaller in CDH cases [4-6].

Retinoids are known to play a critical role not only during lung morphogenesis [7], but also during nephrogenesis [8,9]. Recent studies suggest that retinoid signaling pathway may be disrupted in the nitrofen model of CDH [10]. Therefore, the aim of this study was to investigate the effects of pulmonary hypoplasia on renal development, to test the previously mentioned hypothesis of renal enlargement in CDH, and to evaluate the retinol status of kidneys in the nitrofen model of CDH.

1. Materials and methods

Adults Sprague-Dawley rats were mated overnight. Twelve hours later the presence of spermatozoids in the vaginal smear was verified and this was considered as gestational day 0. Pregnant female rats were then randomly divided into 2 groups. Animals in the first group (n = 8) received intragastrically 100 mg of nitrofen (Wako Chemicals, Osaka, Japan) dissolved in 1 mL of olive oil on day 9.5 of gestation, whereas those in the second group (n = 10) received only vehicle. Gestation was resumed until day 21, in which the rats were sedated with isofluorane and then killed by an intracardiac injection of sodium pentobarbital. The fetuses were then recovered by cesarean delivery; they were inspected and weighed in a precision balance (Ohaus Analytical Plus, Ohaus Corp, Pine Brook, NJ). Under a dissecting microscope (Leica S8 APO, Leica Microsystems, Wetzlar, Germany), the anterior thoracoabdominal wall, the liver, and the gut were removed to expose the lungs, diaphragm, and kidneys. The diaphragm was carefully inspected for the presence of a hernia. Fetuses with diaphragmatic defects were defined as the CDH group, whereas the fetuses exposed to nitrofen with an intact diaphragm represented the nitrofen without CDH group. The control group consisted of animals that only received olive oil. Both lungs were dissected and weighed separately. The kidneys and urinary tracts were carefully observed in situ for the presence of ureterohydronephrosis or other structural urinary malformations. Three fetuses (4%) from both nitrofen groups with urologic malformations were excluded from this study. Only kidneys with a normal aspect were dissected, weighed, and processed for further studies. They were either snap frozen in liquid nitrogen and then stored at -80°C for subsequent biochemical measurements or fixed in 10% buffered formalin for subsequent tissue processing for histology. DNA and protein contents were measured to assess the cellular number and the cell size. Total DNA and proteins were extracted from lungs and kidneys by using TRI Reagent (Molecular Research Center, Cincinnati, Ohio) according to the manufacturer-recommended protocol. Total DNA concentration was measured using a spectrophotometer (Nano Drop ND-1000, Wilmington, Del), and protein concentration was measured using the bicinchoninic acid method (BCA Protein Assay Kit, Pierce, Rockford, Ill). To facilitate comparisons, total DNA and proteins were normalized for fetal body weight. Total retinol concentration of kidney was analyzed by reverse-phase high-pressure liquid chromatography (HPLC) (Waters, Milford, Mass) on C18 column (Supelco, Bellefonte, Pa) according to a previously described protocol [11]. The proportion of proliferating cells was assessed by immunohistochemistry using Ki-67 antibody (mouse antirat monoclonal MIB5, DakoCytomation, Glostrup, Denmark) in paraffin-embedded section of the kidneys. Eight transversal sections from each right and left kidney were stained and the results expressed are the average of them. A proliferation index was calculated by assessing the proportion of positively stained cells over the total number of cells. This was established using a cell counter tool incorporated in software for image analysis (Image J 1.5 Beta 1, Research Services Branch, National Institute of Mental Health, Bethesda, Md). All numerical data are presented as means $\pm$ SD. The differences between the 3 groups were tested by 1-way analysis of variance. Correlations and regression studies were performed between body and total kidney weight for each group. The differences between right and left kidney weight within groups were tested by paired t test. A P value of less than .05 was considered significant. All statistical tests were performed using a commercially available software package (SPSS 11.0 Statistical Analysis Software, SPSS, Chicago, Ill).

Table 1 Kidney measurements in the nitrofen model of CDH

	Control (n = 69)	Nitrofen without CDH (n = 25)	CHD (n = 40)
Fetal weight (mg)	4311.86 ± 781*	3782.79 ± 495	3690.96 ± 872
Both kidneys (mg)	31.75 ± 8.89**	23.73 ± 5.97	24.23 ± 7.7
Both kidneys (% of body weight)	0.73 ± 0.09**	0.62 ± 0.09	0.65 ± 0.08
Both lungs (mg)	130.36 ± 27.49**	97.81 ± 20.07†	62.78 ± 9.58
Both lungs (% of body weight)	3.02 ± 0.27**	2.52 ± 0.19†	1.78 ± 0.52
Total DNA (μg)	110.34 ± 42.15*	64.71 ± 20.28	80.58 ± 35.65
DNA per kidney (μg/mg)	3.43 ± 1.05	3.06 ± 1.19	3.59 ± 1.26
Total protein (μg)	1287.48 ± 312.52	993.2 ± 276.62	1145.59 ± 500.36
Protein per kidney (μg/mg)	47.38 ± 6.93	43.95 ± 6.79	49.76 ± 11.12
Protein/DNA (μg/μg)	16.16 ± 7.02	16.22 ± 6.85	15.12 ± 5.98
Retinol per kidney (μg/g)	2.53 ± 0.61**	1.28 ± 0.11	1.35 ± 0.24
Proliferating cells (% of total cells)	47.64 ± 5.76	47.96 ± 6.01	50.43 ± 8.81

* $P < .01$ vs nitrofen without CDH and CDH groups.
** $P < .001$ vs nitrofen without CDH and CDH groups.
† $P < .001$ vs control and CDH group.

The Department of Health and Children approved all the animal experiments (license no. B100/3530) under the Cruelty to Animals Act, 1876; as amended by European Communities Regulations 2002.

2. Results

A total of 134 fetuses from 18 rats were studied. There were 69 fetuses in the control group, 25 fetuses in the nitrofen without CDH group, and 40 fetuses in the CDH group. No malformations of the diaphragm were seen in the control group. All nitrofen-exposed fetuses were significantly smaller than the control ones and there were no differences in the fetal weight between the CDH group and the nitrofen without CDH group (Table 1). Because we found no differences between right and left kidney weights within each group (control right kidney, 16.1 ± 4.6 mg; control left kidney, 15.65 ± 4.43 mg, P = not significant [NS]; nitrofen without CDH, right kidney, 11.82 ± 2.91 mg; nitrofen without CDH, left kidney, 11.92 ± 3.26 mg, P = NS; CDH, right kidney, 12.17 ± 4.32 mg; CDH, left kidney, 12.06 ± 3.56 mg, P = NS), we considered the weights of both kidneys for the following analysis. The weights of both kidneys were significantly decreased in both nitrofen-exposed groups compared with that of the controls, and when these raw data were converted into both kidneys–body weight ratio, kidneys in both nitrofen without CDH and CDH groups were still significantly smaller than the control ones (Table 1). Weights of both lungs were significantly decreased in nitrofen-exposed groups than those of control group and the ones in the CDH group were smaller than the ones in the nitrofen without CDH group (Table 1). Regression of kidney weight on body weight for the 3 groups is shown in Fig. 1. As expected, a high linear correlation between kidney weight and body weight was found in all the groups: control group, $y = 0.0107x - 14.2624$, $r = 0.9382$, $P < .0001$; nitrofen without CDH group, $y = 0.0100x - 13.9047$, $r = 0.8891$, $P < .0001$; and CHD group, $y = 0.0081x - 5.5577$, $r = 0.9298$, $P < .0001$. No differences could be observed between the correlation coefficient of the 3 groups (control vs nitrofen, no CDH, $z = 1.0048$, $P = .315$; control vs CDH, $z = 0.2802$, $P = .7793$; nitrofen vs CDH, $z = -0.7353$, $P = .4621$). There was no evidence of renal enlargement in the CDH group, but rather a linear correlation with body weight suggesting kidney growth retardation. Kidney total DNA was significantly reduced in both nitrofen-exposed groups, but the DNA concentration was the same in all 3 groups (Table 1). Total amount of proteins, protein concentration, and the amount of protein per microgram of DNA remained the same in the 3 groups (Table 1). Retinol concentration was significantly lower in both nitrofen-exposed groups compared with the

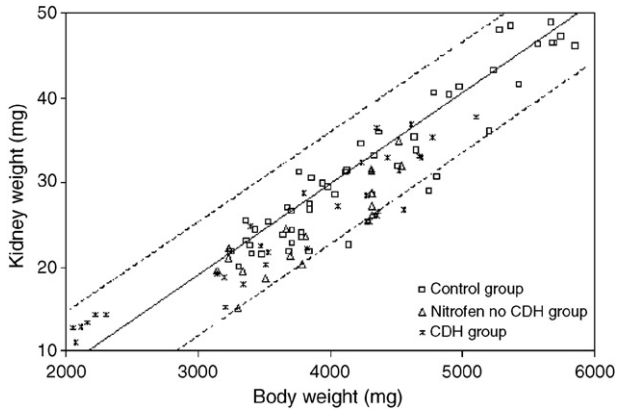

Fig. 1 Regression of kidney weight on body weight for the control ($y = 0.0107x - 14.2624$, $r = 0.9382$, $P < .0001$), nitrofen without CDH ($y = 0.0100x - 13.9047$, $r = 0.8891$, $P < .0001$), and CHD groups ($y = 0.0081x - 5.5577$, $r = 0.9298$, $P < .0001$). A high linear correlation between kidney weight and body weight was found in all the groups. Regression line of the control group is shown. Dispersion of the control group is represented by the 95% prediction interval. Almost all data points from the CDH group were within the dispersion limits.

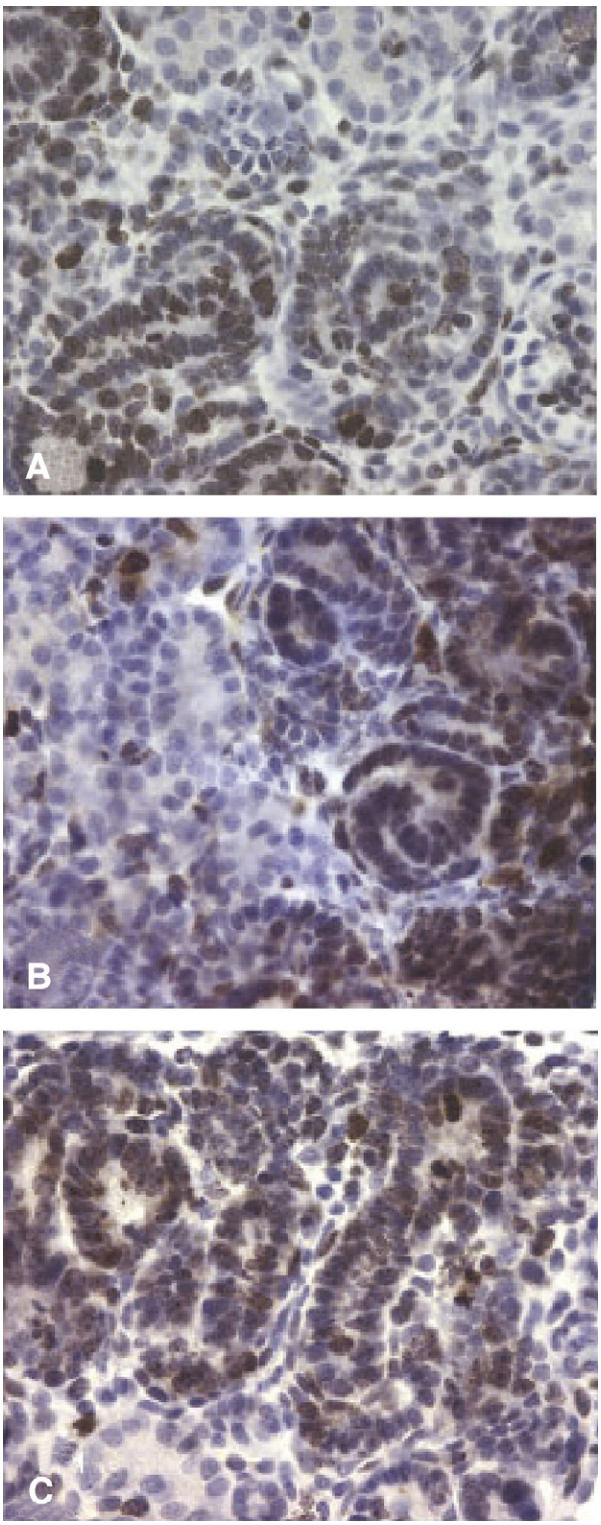

Fig. 2 Representative sections of the subcapsular region of term rat kidney in control (A), nitrofen without CDH (B), and CDH group (C) (original magnification ×400). Proliferating cells are stained with Ki-67 antibody (brown) and show no differences between the groups.

control group. Ki-67–positive cells were located mainly in the subcapsular region of the kidney. The proliferation index was similar in all 3 groups (Table 1 and Fig. 2).

3. Discussion

Our results clearly show that there is no any inverse correlation between lung and kidney development, rejecting a previously proposed hypothesis. Glick et al [2] proposed an integrated hypothesis to explain renal enlargement associated to lung hypoplasia and polyhydramnios seen in severe cases of CDH. According to their hypothesis, in utero growth of the lungs could be stimulated by a pulmonary growth factor produced by the kidneys and that there could be a feedback signal from the lungs to the kidneys by a pulmonary-derived renotropin. Theoretically, pulmonary hypoplasia associated with CDH would maximally stimulate the release of pulmonary-derived renotropin, resulting in renal enlargement and secondary polyhydramnios. They speculated that the pulmonary growth factor, if isolated, could be used therapeutically for fetuses with pulmonary hypoplasia. Glick et al [2] and Hosoda et al [3] found the kidneys to be enlarged in an autopsy study of patients with CDH. They created a surgical model of CDH in lambs in which they not only showed that the kidneys were enlarged, but that they were hyperplastic by quantifying the amount of DNA and proteins on them [3]. However, these authors failed to provide further evidence, and neither pulmonary-derived renotropin nor pulmonary growth factor was ever isolated. Moreover, other investigators later reported no correlation between kidney and lung weight [4-6]. Tovar et al [5] studied the kidneys in the nitrofen model of CDH and found them to be slightly smaller in the nitrofen-exposed animals compared with controls, although not significant. Recently, Rittler et al [6] reported an autopsy study performed on numerous CDH cases. They included the same number of nonmalformed controls matched by weight. After excluding multiply malformed infants and cases with renal anomalies, they found the kidney weight-to-body weight ratio to be significantly lower in CDH cases than in controls. Our present findings in the nitrofen model of CDH are remarkably similar to what Rittler et al [6] found in human autopsies, further supporting the nitrofen model as a good animal model for CDH. We found not only the raw kidney weight to be decreased in both nitrofen-exposed groups compared with the control group, but also to be significantly decreased if converted into kidney weight-to-body weight ratio. As expected, we found a direct linear correlation between the body weight and kidney weight for all the groups. The decreased total DNA in both nitrofen-exposed groups reflects a reduced cell number; however, this reduction was proportional to the reduction in renal mass as the concentration of DNA per kidney remained the same in all 3 groups. The concentration of proteins per kidney and the amount of protein per microgram of DNA

also remained the same for the 3 groups, reflecting a preservation of cell size. These findings suggest that the kidneys are smaller in this model because of hypoplasia. It is interesting to note that we found no differences in kidney weight, DNA and protein concentration, and proliferative activity between nitrofen without CDH and CDH groups. These findings rule out any possible direct stimulus from the lungs to the kidneys because, in that case, we should have found a marked difference in the measured parameters in the kidneys from the CDH group. A variable rate of ureterohydronephrosis has been reported in the nitrofen model of CDH [12-14]. We believe the inclusion of such cases could distort the actual kidney-to-body weight relationship as other authors have also suggested [6]. For that reason we excluded these cases from the present study.

It has been proposed that nitrofen could act by interfering in the retinoic acid pathway [10], and it has recently been demonstrated that the incidence of CDH can be dramatically reduced if retinoic acid is given to pregnant rats along with nitrofen [15]. Retinoids are known to play a critical role not only during lung morphogenesis [7], but also during nephrogenesis [8,9]. Retinoids regulate embryonic kidney patterning through control of Ret expression, which modulates ureteric bud branching morphogenesis [8]. Our study demonstrates that retinol concentration is significantly reduced in kidneys in both nitrofen-exposed groups. These findings suggest that nitrofen, by interfering in the retinoic acid pathway, could be the common mechanism in the pathogenesis of lung and kidney hypoplasia.

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