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Oligohydramnios Decreases Platelet-Derived Growth Factor Expression in Fetal Rat Lungs

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Key Words
Alveolarization · Elastin · Pulmonary hypoplasia

Abstract
Objective: To evaluate the effects of experimental oligohydramnios on lung growth, expression of platelet-derived growth factor (PDGF) and its receptors, and lung morphology in fetal rats. ***Methods:*** On day 16 of gestation, we anesthetized timed pregnant Sprague-Dawley dams and punctured uterine wall and fetal membranes of each uterine sac which resulted in oligohydramnios. The fetuses in the opposite uterine horn served as controls. On days 19 and 21 of gestation, the fetuses were delivered by cesarean section and weighed, and the lungs were dissected free and weighed. ***Results:*** Rats exposed to oligohydramnios exhibited significantly lower lung/body weight ratios on days 19 and 21 of gestation and significantly lower radial saccular counts on day 21 of gestation than did the control rats. Lung PDGF-A and PDGF-B gene and protein expression and elastin level were significantly decreased in rats exposed to oligohydramnios on days 19 and 21 of gestation. The PDGF receptor alpha and beta gene expression levels were significantly decreased in rats exposed to oligohydramnios on day 19 of gestation. ***Conclusion:*** A decreased PDGF expression may be important in the pathogenesis of oligohydramnios-induced pulmonary hypoplasia and suggests that supplementation may provide useful therapeutic strategies.

Introduction

Pulmonary hypoplasia is common in the perinatal period and is a significant cause of death in newborn infants [1]. Oligohydramnios is one of the most common associated abnormalities. Oligohydramnios may retard fetal lung growth and results in pulmonary hypoplasia in experimental animals and in human fetuses, with prolonged rupture of the membrane [2–4]. Platelet-derived growth factor (PDGF) is important for alveolarization of the normally developing lung [5]. PDGFs are homodimers or heterodimers consisting of two distinct polypeptide chains (A and B) which can be dimerized via sulfhydryl bridges to form three bioactive isoforms (AA, BB, and AB) [6]. Physical forces are important in regulating fetal lung growth and maturation [7, 8]. The main physical force that the lung experiences is stretching induced by lung fluid in the airspaces during normal lung development [9]. The fluid maintains the lungs in an expand-

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ed state and provides the tissue with the mechanical stretching that is necessary for normal lung development [10]. Mechanical stretching may increase growth factor expression through intracellular signal transduction pathways [11]. PDGD-A and its receptor are essential in lung elastogenesis and alveolarization [12]. It has been demonstrated that mechanical strain increases the PDGF production and activates the PDGF receptors in vascular smooth muscle cells [13, 14]. Little is known about the effects of oligohydramnios on lung PDGF and elastin in vivo. We hypothesized that oligohydramnios causes a decrement in lung distension and induces a smaller amount of mechanical stretch-induced PDGF activation and retarded lung development. Understanding the factors that are associated with oligohydramnios will enhance the development of therapies capable of promoting lung growth in such diseases, for which currently no specific treatment is available.

Table 1. Oligonucleotide sequences of the primers used

Primer	Sequence 5′→3′	Product size, bp
PDGF-A		
Forward	AGGTGAGGTTAGAGGAGCAC	318
Reverse	TCGCTCTCTGTGACAAGG	
PDGF-B		
Forward	CACATTCTGGAGTCGAGTCG	426
Reverse	TCACCCGAGTTTGAGGTGTC	
PDGFR-α		
Forward	AAGAGAGAGGACGAGACCAT	204
Reverse	ACTTCTGTCTCCACATCACC	
PDGFR-β		
Forward	TACGTGTGAAGGTGTCAGAA	564
Reverse	CAGACTCAATGACCTTCCAT	
Tropoelastin		
Forward	TGGAGCCCTGGGATATCAAG	369
Reverse	GAAGCACCAACATGTAGCAC	
β-Actin		
Forward	TTGTAACCAACTGGGACGATATGG	764
Reverse	GATCTTGATCTTCATGGTGCTAGG	

Materials and Methods

Animals

This study was approved by the Animal Care and Use Committee at Taipei Medical University and was performed with timed pregnant Sprague-Dawley rats. On day 16 of gestation, pregnant dams were anesthetized with pentobarbital (50 mg/kg i.p.). Oligohydramnios was induced as previously described, with minor modifications [15–17]. An abdominal midline incision was made, and the two uterine horns were exposed and kept moist with phosphate-buffered saline. Uterine wall and fetal membranes of each uterine sac in one horn were punctured using a 19-gauge needle which resulted in immediate visible leakage of amniotic fluid and a decrease in the size of the uterine sac. Fetuses in the opposite uterine horn served as controls. The uterus was returned to the abdomen, and the abdominal incision was repaired. On days 19 and 21 of gestation, the dams were anesthetized by pentobarbital (50 mg/kg i.p.), and the fetuses were delivered by cesarean section, killed by pentobarbital (100 mg/kg i.p.) before beginning to breathe, and weighed. The lungs were removed and weighed and the values expressed as percentage of body weight.

Reverse Transcription-Polymerase Chain Reaction

The left lung was ground into powder in liquid nitrogen, and the gene expression was measured with reverse transcription-polymerase chain reaction. Total RNA was extracted using the TRIzol reagent (Invitrogen Life Technologies, Paisley, UK), according to the manufacturer's instructions. Reverse transcription was performed on 3 μg of RNA with an oligo-dT primer and avian myeloblastosis virus reverse transcriptase (Roche Molecular Biochemicals, Indianapolis, Ind., USA). The polymerase chain reactions were carried out with the primers shown in table 1. The polymerase chain reaction products were analyzed by electrophoresis on an agarose gel, stained with ethidium bromide, and photographed. To determine the linear range of the polymerase chain reaction, the intensity of amplified products was plotted against the cycle number. At least five samples on each gestational day were analyzed for each gene in each group and the results expressed as percentage of β-actin.

Western Blot Analysis

Lung tissues were homogenized in Tris buffer with protease inhibitor cocktail tablets. Proteins were separated on SDS-PAGE under a nonreducing condition and electrotransferred to a polyvinylidene difluoride membrane. The blots were incubated for 2 h at room temperature in blocking buffer with mouse monoclonal antibody against PDGF-AA and goat polyclonal antibody against PDGF-BB. After incubation with horseradish-peroxidase-conjugated anti-mouse or anti-goat IgG antibody, the immunoreactive bands were detected by incubation with SuperSignal West Pico Chemiluminescent Substrate reagent (Pierce Biotechnology, Rockford, Ill., USA). The data were normalized to β-actin for each animal.

Determination of Lung Elastin

Lung elastin was dye precipitated and quantified according to the manufacturer's instructions using the Fastin elastin assay (Biocolor, Newtownabbey, Northern Ireland, UK). Briefly, 30 μg of the extracted protein was mixed with 300 μl reagent, and the tubes were shaken for 30 min to allow elastin-dye binding to complete. The unbound dye was removed by centrifugation at 16,000 *g* for 15 min, washed with ethanol, and dissolved in the alkaline dye. Finally, the samples were introduced into a microplate reader, and the absorbance was determined at 550 nm. The calibration curve for the spectrometer had been drawn using the supplied elastin standard. The elastin level in each specimen was obtained as an average of three readings.

Table 2. Body weight, lung weight, and lung/body weight ratio determined on gestational days 19 and 21 in control rats and rats exposed to oligohydramnios

Treatment	n	Body weight g	Lung weight g	Lung/body weight ratio %
Gestational day 19				
Control	16	2.02 ± 0.13	0.08 ± 0.01	3.71 ± 0.10
Oligohydramnios	23	2.08 ± 0.11	0.07 ± 0.00	3.18 ± 0.10***
Gestational day 21				
Control	27	4.10 ± 0.12	0.15 ± 0.01	3.65 ± 0.11
Oligohydramnios	23	3.88 ± 0.20	0.12 ± 0.01**	3.04 ± 0.08***

** $p < 0.01$; *** $p < 0.001$ versus control rats at each gestational age.

Morphological Analysis

The right lungs were removed and fixed in 10% neutral buffered formalin on each gestational day in each group. Serial lung sections were cut at a thickness of 4 μm and stained with hematoxylin and eosin. The number of distal air sacs across the terminal respiratory units was estimated by the radial saccular count method [18]. This assessment was repeated for ten terminal respiratory units in random tissue sections per rat in each group (n = 6).

Immunohistochemistry

Immunohistochemical staining for PDGF-A and PDGF-B was performed on paraffin sections with immunoperoxidase visualization. After deparaffinization in xylene and rehydration in an alcohol series, the sections were first preincubated for 1 h at room temperature in 0.1 M phosphate-buffered saline containing 10% normal goat serum and 0.3% H_2O_2 to block endogenous peroxidase activity and nonspecific binding of antibody before being incubated for 20 h at 4°C with mouse monoclonal antibody against PDGF-AA and goat polyclonal antibody against PDGF-BB. The sections were then treated for 1 h at room temperature with biotinylated anti-mouse and anti-goat IgG. This was followed by reaction with the reagents from an ABC kit (avidin-biotin complex; Vector Laboratories, Burlingame, Calif., USA), according to the manufacturer's recommendations, and the reaction products were visualized by 3,3′-diaminobenzidine and 0.003% H_2O_2 in 0.5 M Tris buffer (pH 7.6), before the sections were mounted on gelatin-coated slides using Permount (Fisher Scientific, Pittsburgh, Pa., USA).

Statistics

The results are presented as mean values ± SEM. Comparisons between control and oligohydramnios groups at equivalent gestational age were made using unpaired Student's t test. Differences were considered significant at $p < 0.05$.

Results

There were 3 and 6 pregnant dams used on days 19 and 21 of gestation, respectively. Control fetuses were all alive at the time of the cesarean section. 3 of 26 (11.5%) and 30 of 53 (56.6%) oligohydramnios-exposed fetuses were dead on days 19 and 21 of gestation, respectively.

Body Weight, Lung Weight, and Lung/Body Weight Ratio

Rats exposed to oligohydramnios exhibited significantly lower lung/body weight ratios on day 19 of gestation and lower lung weights and lower lung/body weight ratios on day 21 of gestation when compared with control rats (table 2).

PDGF and PDGF Receptor (PDGFR) Gene Expression

Rats exposed to oligohydramnios had significantly decreased PDGF-AA and PDGF-BB and PDGFR-α and PDGFR-β gene expression levels on day 19 of gestation (fig. 1a). On day 21 of gestation, rats exposed to oligohydramnios also had a decreased PDGF and PDGFR gene expression, and the values reached statistical significance for PDGF-AA and PDGF-BB only (fig. 1b).

Western Blot Analysis of PDGF

PDGF-AA and PDGF-BB proteins were significantly decreased in fetal lung tissues exposed to oligohydramnios on day 16 and harvested on days 19 (fig. 2a) and 21 (fig. 2b) of gestation.

Immunohistochemistry for PDGF-AA and PDGF-BB

Immunoreactivities of PDGF-AA (fig. 3a) and PDGF-BB (fig. 3b) were detected mainly in airway epithelial and in some mesenchymal cells, and the immunoreactivity was markedly reduced in oligohydramnios-exposed rats on days 19 and 21 of gestation.

Lung Tropoelastin Gene Expression and Elastin Level

The lung tropoelastin gene expression was significantly decreased in rats exposed to oligohydramnios than in

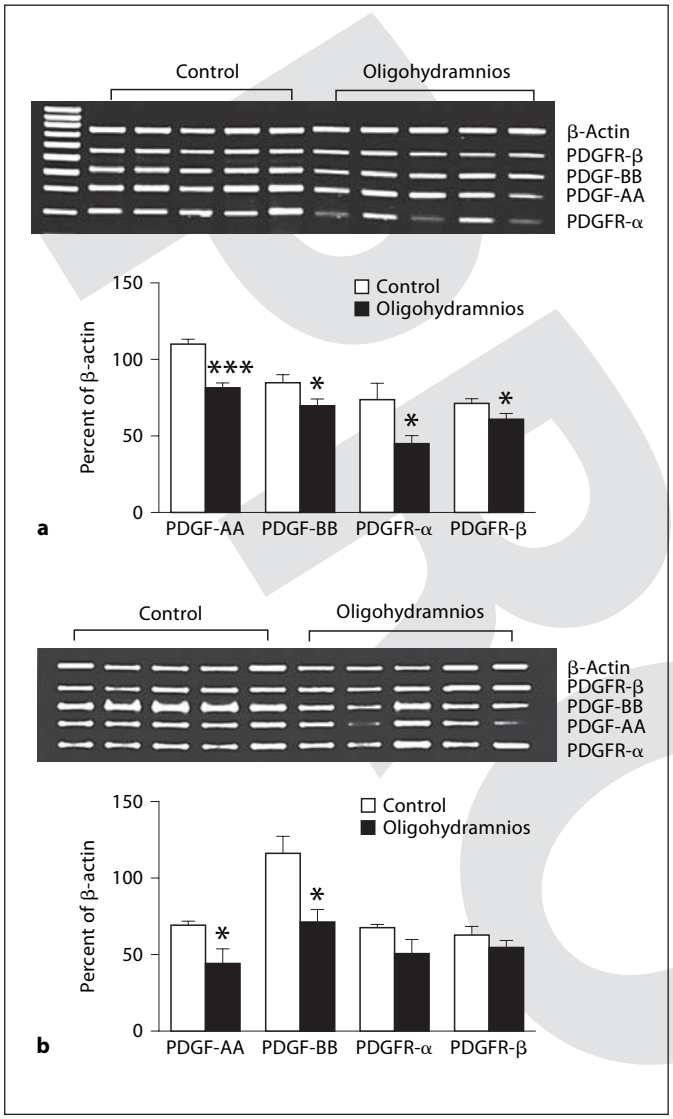

Fig. 1. PDGF and PDGFR gene expression in fetal rat lungs on days 19 (**a**) and 21 (**b**) of gestation. Data were normalized to β-actin for each animal. * $p < 0.05$; *** $p < 0.001$ versus control group.

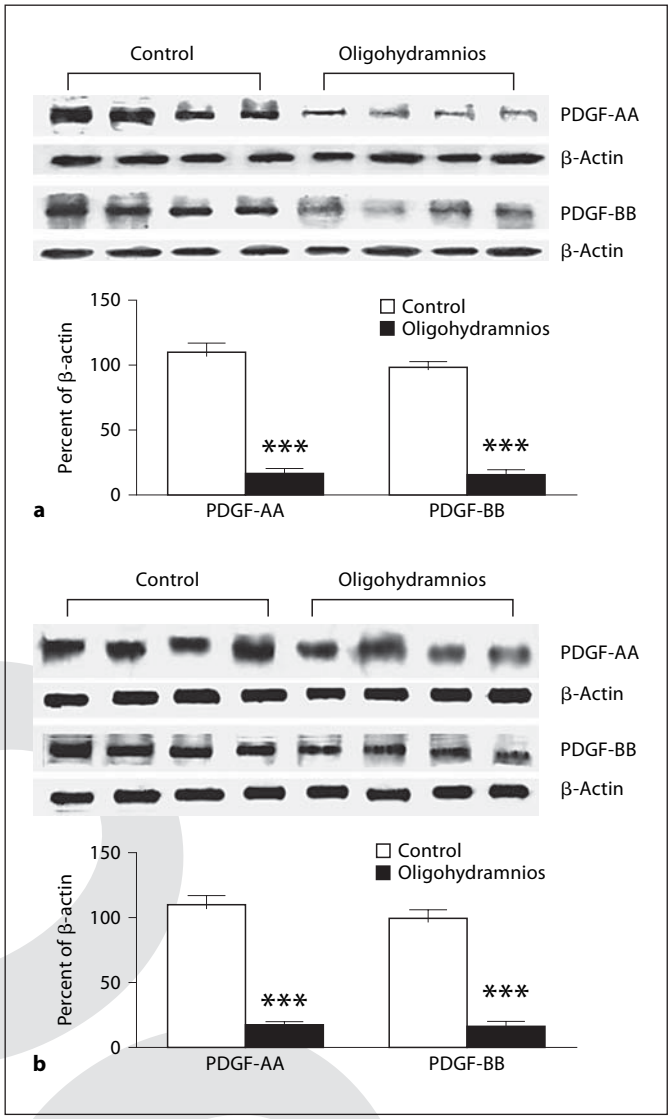

Fig. 2. Representative Western blots and quantitative data determined by densitometry for PDGF-AA and PDGF-BB proteins in fetal rat lungs exposed to oligohydramnios on day 16 and harvested on days 19 (**a**) and 21 (**b**) of gestation. Data were normalized to β-actin for each animal. *** $p < 0.001$ versus control group.

control rats on days 19 and 21 of gestation (fig. 4). The lung elastin levels (μg/mg protein) were significantly lower in the rats exposed to oligohydramnios when compared to control rats on days 19 (371.7 ± 13.1 vs. 430.9 ± 10.1, $p < 0.05$) and 21 (334.4 ± 32.8 vs. 483.8 ± 12.0, $p < 0.01$) of gestation (fig. 5).

Histology

Examination of random fields under a light microscope revealed fewer epithelial tubules in rats exposed to oligohydramnios as compared with the control rats on day 19 of gestation. Lung section depicts fewer saccules in oligohydramnios rats on day 21 of gestation (fig. 6a). The radial saccular count was significantly lower in rats exposed to oligohydramnios than in control rats on day 21 of gestation (fig. 6b).

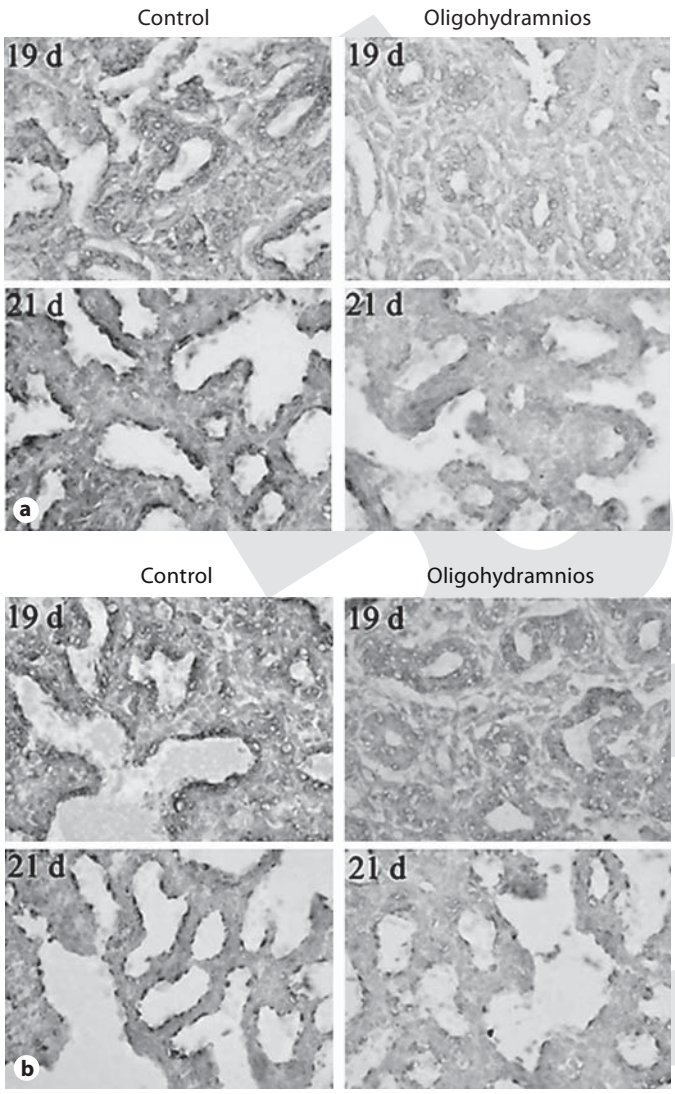

Fig. 3. Immunohistochemistry for PDGF-AA (**a**) and PDGF-BB (**b**) in fetal rat lung sections on days 19 and 21 of gestation. Positive staining is shown as black. ×400.

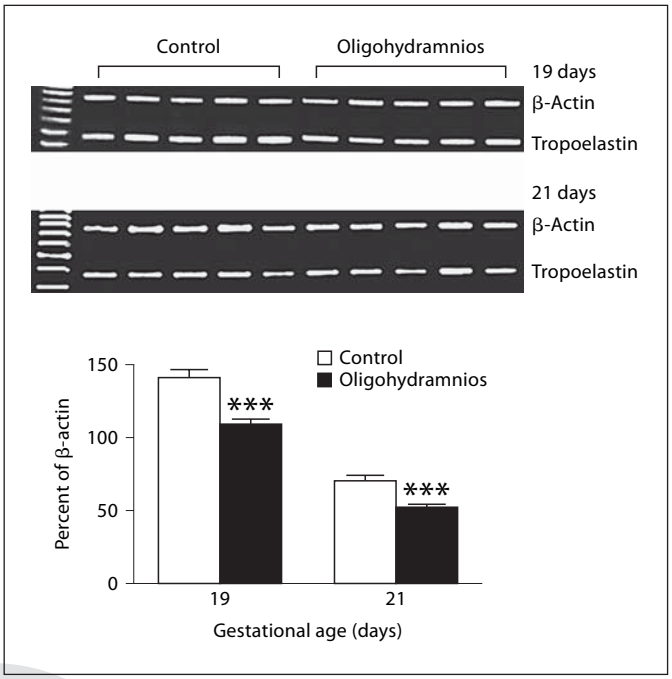

Fig. 4. Tropoelastin gene expression in fetal rat lungs on days 19 and 21 of gestation. Data were normalized to β-actin for each animal. *** $p < 0.001$ versus control group.

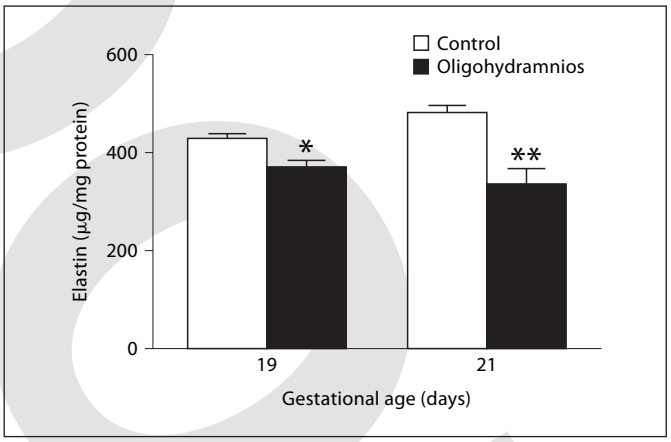

Fig. 5. Lung elastin levels in fetal rat lungs on days 19 and 21 of gestation. * $p < 0.05$; ** $p < 0.01$ versus control group.

Discussion

Moessinger et al. [15] induced oligohydramnios from days 15 to 21 of gestation and found a significant reduction in lung weight and lung/body weight ratio as well as reduced DNA per lung and lung protein/DNA ratio. Kitterman et al. [16] punctured fetal rat amniotic sacs on day 16 of gestation and found a significant reduction in lung weight, lung/body weight ratio, and total DNA and protein contents on day 21 of gestation. Blachford and Thurlbeck [17] performed amniocentesis on day 16 of gestation in fetal rats and found significant reductions in lung weight and lung/body weight ratio and comparable DNA and protein concentrations on day 21 of gestation. The general manifestations of retarded lung growth induced

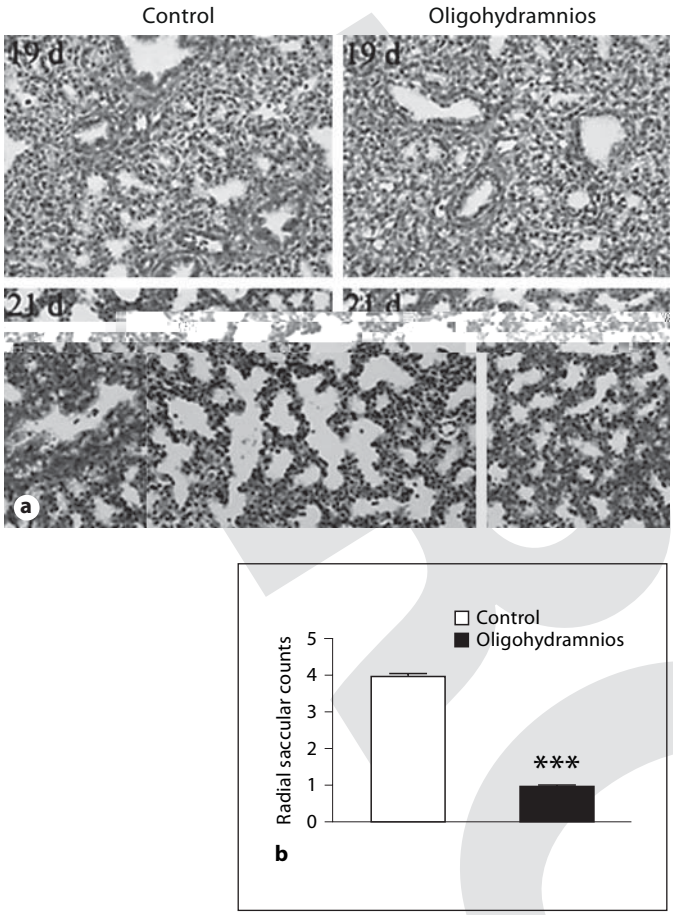

Fig. 6. a Representative histological appearance of HE-stained lungs. ×200. **b** Radial saccular counts in fetal rats on day 21 of gestation (n = 6). *** $p < 0.001$ versus control group.

by oligohydramnios are reduced lung weight and reduced lung/body weight ratio. In the present study, we induced oligohydramnios on day 16 of gestation and found significant reductions in lung/body weight ratio and lung development on days 19 and 21 of gestation. The magnitude of lung growth retardation was greater on day 21 of gestation. Therefore, oligohydramnios did produce pulmonary hypoplasia based on lung/body weight ratio and histological findings.

Alveolarization predominantly occurs within the first 2 weeks of postnatal life in rats. In this study, alveolarization was measured in the fetal rats on day 21 of gestation during the saccular stage. Consequently, the term 'saccules' replaced 'alveoli' for the radial alveolar count. We found that maternal oligohydramnios created on day 16 of gestation induced pulmonary hypoplasia with decreased lung/body weight ratio on days 19 and 21 of gestation and decreased the radial saccular count on day 21 of gestation.

PDGF is a powerful stimulator of fibroblast chemotaxis and proliferation [19, 20]. Han et al. [21, 22] reported that both PDGF and its receptors are present in fetal rat lungs. Our study showed that rats exposed to oligohydramnios exhibited downregulation of PDGF and reduced lung development. These results were compatible with results reported by Souza et al. [23, 24] who used antisense oligonucleotides in embryonic rat lung explant cultures and found that PDGF plays critical roles in early lung growth and branching morphogenesis.

The mechanism that downregulates the expression of PDGF in the setting of oligohydramnios-induced pulmonary hypoplasia is not clear. Two major stimuli to fetal lung growth result from stretching due to intermittent and repetitive fetal breathing movements and a constant distending pressure, when fetal breathing movements are absent [9]. The constant distending pressure is produced by the secretion of lung fluid and the resistance to outflow in the upper airways [25]. In the fetus, the lungs are filled with a fluid that is secreted from the pulmonary epithelium into the potential airspaces and leaves the lungs via the trachea. Oligohydramnios does not influence fetal breathing movements, but it decreases the volume of fluid within the potential airways and airspaces [3, 26]. The fluid maintains the lungs in an expanded state and provides mechanical stretching for the tissue that is necessary for normal lung development [10]. Mechanical stretching increases growth factor expression through intracellular signal transduction pathways [11]. Elastin is an important structural component of alveolar wall and conducting airways and allows expansion and recoil of the lung that are essential to its mechanical performance [27]. There is a strong temporal and spatial relationship between the expression of elastin and the development of terminal airspaces. In the developing lung, elastin is deposited in the mesenchyme surrounding the developing distal airways before alveolarization and at the apex of the secondary septal crests during the process of alveolarization [28]. In this study, we found that oligohydramnios decreased the elastin levels in hypoplastic fetal rat lungs. This result is consistent with the observation of Haider et al. [29] who found that elastic tissue was missing in hypoplastic human fetal lungs associated with oligohydramnios. Joyce et al. [30] found that sustained reductions in lung expansion by tracheal drainage decreased the elastin expression. The above studies further sup-

port our suggestion that pulmonary hypoplasia associated with oligohydramnios results from a reduction in fetal lung expansion.

In conclusion, these results show that maternal oligohydramnios during late gestation decreases the expression of PDGF and its receptors, decreases elastin gene expression and concentration, and arrests fetal lung development in rats. These data suggest that a decreased PDGF expression may be important in the pathogenesis of oligohydramnios-induced pulmonary hypoplasia. The underlying mechanism is unclear, and its elucidation may provide useful therapeutic strategies.

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