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# Gene expression analysis in hypoplastic lungs in the nitrofen model of congenital diaphragmatic hernia

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**Abstract**

**Background:** Pulmonary hypoplasia and persistent pulmonary hypertension are the main causes of mortality and morbidity in newborns with congenital diaphragmatic hernia (CDH). Nitrofen is well known to induce CDH and lung hypoplasia in a rat model, but the mechanism remains unknown. To increase the understanding of the underlying pathogenesis of CDH, we performed a global gene expression analysis using microarray technology.

**Methods:** Pregnant rats were given 100 mg nitrofen on gestational day 9.5 to create CDH. On day 21, fetuses after nitrofen administration and control fetuses were removed; and lungs were harvested. Global gene expression analysis was performed using Affymetrix Platform and the RAE 230 set arrays. For validation of microarray data, we performed real-time polymerase chain reaction and Western blot analysis.

**Results:** Significantly decreased genes after nitrofen administration included several growth factors and growth factors receptors involved in lung development, transcription factors, water and ion channels, and genes involved in angiogenesis and extracellular matrix. These results could be confirmed with real-time polymerase chain reaction and protein expression studies.

**Conclusions:** The pathogenesis of lung hypoplasia and CDH in the nitrofen model includes alteration at a molecular level of several pathways involved in lung development. The complexity of the nitrofen mechanism of action reminds of human CDH; and the picture is consistent with lung hypoplasia and vascular disease, both important contributors to the high mortality and morbidity in CDH. Increased understanding of the molecular mechanisms that control lung growth may be the key to develop novel therapeutic techniques to stimulate pre- and postnatal lung growth.



Congenital diaphragmatic hernia (CDH) was first described by Bochdalek in 1848. The incidence of CDH is 1 of 2500 to 3500 births. It remains one of the most life-threatening causes of severe respiratory failure in the neonate. It occurs on the left in 85% to 90% of the cases. The mortality rate remains high, 30% to 50%, because of pulmonary hypoplasia and persistent pulmonary hypertension; and also, the morbidity rate is still high in survivors [1,2].

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It was classically believed that failure to close the pleuroperitoneal canals resulted in a diaphragmatic defect, allowing intraabdominal viscera to enter the thoracic cavity, compressing the lungs and resulting in pulmonary hypoplasia. However, recent data from experimental studies support a more complex molecular and cellular hypothesis based on concomitant abnormalities of diaphragm and lung development [3,4]. As a result, infants with CDH are born with smaller lungs (pulmonary hypoplasia) and an associated decrease in the cross-sectional area of the pulmonary vasculature (pulmonary vascular hypoplasia). Congenital diaphragmatic hernia is also associated with delayed lung maturation (lower number of airway and vascular generations and decreased radial alveolar count) and alterations in pulmonary vascular structure (greater muscularity of the peripheral arteries) [5].

The etiology of CDH remains largely unclear; and as with any disease of unknown etiology, the ability to investigate the pathogenesis and pathophysiology is dependent on the availability of appropriate animal models. In CDH research, there are 2 principal types of animal models: surgically created [6] and teratogen induced [7,8], dietary and genetic models of diaphragmatic defects [8]. An important drawback in the surgically created CDH in lambs or rabbits models is that the diaphragmatic defect is created relatively late in gestation, unlike the human disease. A number of chemicals have been shown to induce CDH in rodents. One of these, nitrofen (2,4-dichloro-4′-nitrodiphenyl ether), is an herbicide that administrated to pregnant rodents as a single oral dose at a specific point in gestation results in multiple teratogenic effects in the fetus, including diaphragmatic defects, pulmonary hypoplasia, pulmonary immaturity, and pulmonary vascular anomalies [9,10]. In this model, the diaphragmatic defect is produced during an early stage of lung development and more closely reflects the human disease.

An increasing number of growth and transcription factors have previously been reported to have an altered expression in this model, such as decreased vascular endothelial growth factor (VEGF) on gestational days 15 to 18 [11]; fibroblast growth factor (FGF) 7, 10, and 18 [12,13], homeobox b-5 [14], and thyroid transcription factor 1 at term [15]; Wingless/integrated 2 and 7 on gestational day 13 [16]; GATA 4 and 6 on gestational day 15 [17]; and Sonic hedgehog [18], insulin-like growth factor I [19], phospholipids, surfactant proteins A, B, and C [20], and ion and water channels [21-23] at term, all being decreased after nitrofen. An increased expression of matrix metalloproteinase 9 (MMP-9) has been reported [24], as well as an alteration of the retinoid acid pathway at different levels [25-28]. The lungs of rodents with nitrofen-induced CDH have also been shown to have increased expression of intercellular adhesion molecule 1 and vascular cell adhesion molecule 1 [29], increased angiotensin-converting enzyme activity [30], decreased endothelial isoform of nitric oxide synthase (eNOS) expression [31], increased endothelin-1 and endothelin A vasoconstrictor receptor [32], and altered $K^+$ channel expression [33], which might contribute to increased pulmonary vascular tone in the teratogenic model of CDH.

The understanding of the pathogenesis of the nitrofen effects in lung development is still incomplete. To increase the understanding of the biological mechanisms involved in the teratogenic effects of nitrofen in lung development, we performed genomewide gene expression analysis using the Affymetrix GeneChip Microarray Platform (Santa Clara, CA).

## 1. Material and methods

### 1.1. Experimental CDH

Time-mated Sprague-Dawley rats (Scanbur B&K, Sollentuna, Sweden) were given 100 mg nitrofen (Fluka, Deisenhofen, Germany) dissolved in 1 mL olive oil by gavage on gestational day 9.5 [7,9,34]. Control animals were given olive oil only. After intraperitoneal injection of pentobarbital (15 mg/kg body weight) on day 21(term 22), the fetuses were removed by caesarian section and weighed. The sternum was removed under a dissecting microscope, and the thoracic cavity was inspected. Only fetuses with diaphragmatic hernia were chosen, and both lungs were excised and weighed. The fetal lungs were then frozen at −70°C. The same procedure was used to remove the lungs of control fetuses.

### 1.2. RNA isolation and hybridization

To ensure the reproducibility of the microarray experiments and to address the potential biological individual variability, the assays were performed in biological triplicates using 3 separate messenger RNA (mRNA) preparations from 3 hypoplastic-CDH lungs after nitrofen and 3 mRNA preparations from 3 normal nonmanipulated fetal lungs, as previously described [35]. Briefly, total RNA from lungs was extracted using Trizol and further purified. The RNA quality was tested using the Agilent 2100 Bioanalyzer (Santa Clara, CA). If high quality was confirmed, double-stranded complementary DNA (cDNA) was synthesized by reverse transcription of purified mRNA. The cDNA was used as a template for the generation of biotin-labeled in vitro transcribed complementary RNA (cRNA), using biotinylated uridine 5′-triphosphate (UTP and citidine 5′-triphosphate (CTP) ribonucleotides. The biotin-labeled anti-sense cRNA was then purified, fragmented, and hybridized to the Affymerix RAE 230 set chip arrays according to the protocol recommended by the supplier. The Affymerix RAE 230 set chip arrays contains approximately 28,000 well-substantiated rat genes, 30,200 transcripts, and variants represented on 31,000 probe sets. After extensive washing, the arrays were stained with streptavidin-phycoerythrin followed by a second staining with biotin-labeled anti-streptavidin antibody and a repeated streptavidin-phycoerythrin staining to