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The effect of in vitro tracheal occlusion on branching morphogenesis in fetal lung explants from the rat nitrofen model of congenital diaphragmatic hernia

Jeremy R. Grushka[a], Saleh Al-Abbad[a], Robert Baird[a], Pramod Puligandla[a], Feige Kaplan[b,c], Jean-Martin Laberge[a,*]

[a]*Division of Pediatric Surgery, The Montreal Children's Hospital, Montreal, Quebec, Canada*
[b]*Department of Pediatrics Montreal Children's Hospital Research Institute, McGill University, Montreal, Quebec, Canada*
[c]*Department of Human Genetics, Stewart Biology Building, McGill University, Montreal, Quebec, Canada*

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Abstract

Background/Purpose: Fetal tracheal occlusion (TO) has been investigated as a treatment option for lung hypoplasia secondary to congenital diaphragmatic hernia. Tracheal occlusion has been shown to accelerate lung growth, but its effect on bronchial branching is unknown. In this study, we characterize the effects of in vitro TO on bronchial branch development in fetal lung explants derived from the nitrofen rat model of congenital diaphragmatic hernia.

Methods: Rat dams were gavaged nitrofen on gestational day 9.5, and fetal lungs were harvested for explant culture on gestational day 14 (term, 22 days). Four experimental groups were investigated, with TO performed ex vivo using cautery: control, control + TO, nitrofen, and nitrofen + TO. Explants were incubated for 72 hours. Representative photographs were taken at 0, 24, 48, and 72 hours from the time of culture, and the number of distal branches was counted for each explant. The Student *t* test was used to compare distal branch measurements.

Results: A minimum of 12 fetal lung explants were cultured for each group. By 24 hours, all explants undergoing TO had more branch iterations than explants that did not. Moreover, TO in nitrofen-exposed explants increased bronchial branching to control levels by 24 hours in culture.

Conclusion: Our results suggest that TO at day 14 increases branching in normal and nitrofen-exposed lung explants. In addition, TO increases airway branching in nitrofen-exposed explants to control levels suggesting that early TO reverses the lung hypoplasia seen in this model.

Congenital diaphragmatic hernia (CDH) affects approximately 1 in 2200 total births, including stillbirths [1,2]. Despite advances in neonatal care, the mortality rate of isolated CDH remains 20% to 40% [3,4]. Furthermore, high-risk neonates with CDH, such as those whose liver is within the hernia, may experience mortality rates exceeding 50% [5,6]. Congenital diaphragmatic hernia is associated with lung hypoplasia, pulmonary hypertension, and surfactant deficiency [7-9], all of which contribute to the acute

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* Corresponding author. Tel.: +1 514 412 4498; fax: +1 514 412 4289.
E-mail address: jean-martin.laberge@muhc.mcgill.ca (J.-M. Laberge).

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respiratory insufficiency observed in these neonates. Histologically, CDH-affected lungs demonstrate a marked reduction in bronchial divisions that is more severe on the ipsilateral side of the hernia [10]. Currently, most infants with CDH are managed with postnatal intensive care therapy followed by delayed repair of the defect, but prenatal intervention is being investigated as a tool to improve the fate of high-risk fetuses.

The concept of fetal tracheal occlusion (TO) to reverse lung hypoplasia was introduced in the early 1990s as a potential treatment for CDH [11]. Tracheal occlusion in utero has been shown to accelerate lung growth, reversing the morphologic appearance of lung hypoplasia and improving postnatal pulmonary function and compliance in animal models [12-18]. It is unclear, however, whether TO stimulates mature lung growth or induces alveolarization without concomitant bronchial development. This distinction has important clinical ramifications because increased alveolarization may not be sufficient to restore normal lung function. Furthermore, the inability of a recent clinical trial comparing fetal TO and standard postnatal care to demonstrate differences in morbidity and survival may have been because not only of a liberal definition of a "high-risk" CDH but also of the timing at which TO was performed [19,20]. Therefore, the utility of TO in the treatment of high-risk CDH infants remains under investigation but may require earlier application to promote mature lung branching and optimize clinical benefit.

Currently, we are studying lung branching morphogenesis in an established, nitrofen-induced rat model of CDH [21]. Evidence suggests that bronchial branching is inhibited by CDH as reflected by morphometric analyses and the downregulation of molecular markers that are normally active during fetal lung branching [22-25]. We have previously shown that fetal TO in the nitrofen rat model at gestational day 19 (E19) promotes distal airway proliferation but does not reverse the underdevelopment of bronchial branching resulting from CDH [26]. The aim of the current study was to assess the ability of TO, performed at an earlier time point (E14), to promote bronchial branching in control and nitrofen-exposed fetal rats, with or without TO.

1. Materials and methods

1.1. Animal protocol

Ethics approval was obtained from the McGill University animal care committee consistent with Institutional Animal Care and Use Committees regulations (protocol #4663). Time-dated pregnant rats (Charles River, St-Constance, Quebec, Canada) were identified by vaginal semen plug, defined as day 0.5. These animals were transferred to the animal care facility of the Montreal Children's Hospital 5 days before the start of any experimental work to ensure optimal acclimatization. Animals were housed in groups of 2 per cage in standard laboratory conditions and were allowed food and water ad libitum.

Study animals were gavaged nitrofen (2,4-dichlorophenyl *p*-nitrophenyl-ether; Chem Services, West Chester, PA) on E9.5. Nitrofen (100 mg) was dissolved in 1 to 2 mL of olive oil. A rigid metal tube was then inserted into the animal's esophagus to the approximate level of the upper stomach and the olive oil–nitrofen mixture was administered. There was no evidence of reflux or regurgitation after this procedure, and it was well tolerated in all animals. Control animals were gavaged olive oil alone. All chemical handling was done in a fume hood with appropriate safety precautions.

1.2. Organ culture

Rat dams were euthanized by intraperitoneal pentobarbital injection. Control and nitrofen-exposed E14 rat embryos were dissected in phosphate-buffered saline solution (Gibco, Grand Island, NY). Using a dissecting microscope, fetal lung explants were removed en bloc with the fetal trachea and placed on 4.0-μm Nucleopore Track-Etch membranes (Whatman, Piscataway, NJ) and cultured in 1 mL of Dulbecco's modified Eagle medium F-12 (Gibco) supplemented with 50 U/mL penicillin/streptomycin and 10% fetal bovine serum. Dishes were incubated at 37°C and 5% CO_2 for 72 hours. The culture medium was changed at 24-hour intervals.

1.3. TO and airway branching

There were 4 experimental groups: control, control + TO, nitrofen-exposed, and nitrofen-exposed + TO. Before placing the explants in culture, TO was performed using a low-temperature disposable surgical cautery as previously described [27] (Allegiance Healthcare Corporation, McGraw Park, IL). Representative photographs of each explant were taken daily (Fig. 1). The number of distal branches was counted for each lung of the 4 experimental groups, at 0, 24, 48, and 72 hours of culture using a previously described method of counting airway branches [28]. Two individuals counted the airway branches for each lung explant at each time point. One individual was blinded to both the form of treatment that the rat received and whether or not TO was performed, whereas the other individual was not blinded. The mean number of airway branches was then used for subsequent analyses. These results were pooled within each group and compared using a nonpaired Student *t* test (DATA Pro software; Microsoft). $P < 0.05$ was considered significant in all cases.

2. Results

Four groups were analyzed with a minimum of 12 explants in each group: control (n = 16), control + TO (n = 17), nitrofen-exposed (n = 22), and nitrofen-exposed + TO (n = 12). Results are presented in Table 1.