Original article

Antenatal use of bosentan and/or sildenafil attenuates pulmonary features in rats with congenital diaphragmatic hernia

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Background: **Lung hypoplasia, pulmonary persistent hypertension of the newborn and its morphological changes are the main features in congenital diaphragmatic hernia (CDH). This study was undertaken to investigate if antenatal use of sildenafil and/or bosentan attenuates vascular remodeling, promotes branching, and improves alveolarization in experimental nitrofen-induced CDH.**

Methods: **Nitrofen (100 mg) was gavage-fed to pregnant rats at post conception day (PCD) 9 to induce CDH. The rats were randomized to 5 groups: 1) control; 2) nitrofen; 3) nitrofen+sildenafil 100 mg/kg per day at PCD 16-20; 4) nitrofen+bosentan 30 mg/kg per day, at PCD 16-20, and 5) nitrofen+bosentan+sildenafil, same doses and administration days. After cesarean delivery, the offsprings were sacrificed. The diaphragmatic defect and pulmonary hypoplasia were identified, and the lungs were dissected. Arterial wall thickness, bronchiolar density and alveolarization were assessed.**

Results: **The offsprings with CDH were characterized by severe pulmonary hypoplasia (lung weight-to-body weight ratio: 0.0263 [95% confidence interval (CI) 0.0242-0.0278)] in the nitrofen group versus 0.0385 (95% CI 0.0355-0.0424) in the control group (*P*=0.0001). Pulmonary arterial wall thickness was decreased to 3.0 (95% CI 2.8-3.7) μm in the nitrofen+sildenafil group versus 5.0 (95% CI 4.1-4.9) μm in the nitrofen group (*P*=0.02). Terminal bronchioles increased to 13.7 (95% CI 10.7-15.2) μm in the nitrofen+bosentan group in contrast to 8.7 (95% CI 7.2-9.4) μm in the nitrofen group (*P*=0.002). More significant differences (*P*=0.0001) were seen in terminal bronchioles in the nitrofen+sildenafil+bosentan group than in the nitrofen group [14.0 (95% CI 12.5-15.4) μm versus 8.5 (95% CI 7.1-9.3) μm]. Pulmonary arterial wall thickness was also decreased in the former group.**

Conclusions: **In this rat model, antenatal treatment with sildenafil attenuates vascular remodeling. Bosentan promotes the development of terminal bronchioles in nitrofen-induced CDH.**

World J Pediatr 2014;10(4):354-359

Key words: **antenatal treatment;**
bosentan;
congenital diaphragmatic hernia;
pulmonary hypoplasia;
sildenafil

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doi: 10.1007/s12519-014-0512-y

Introduction

Congenital diaphragmatic hernia (CDH) is a severe condition which affects 1 in 2000 to 3000 live births. Despite advances in neonatal care, CDH is associated with a high risk of mortality and morbidity.[1] The clinical course of affected newborns is defined by the extent of respiratory failure at birth resulting from pulmonary hypoplasia, reduced airway branching, and decreased surfactant. Extensive muscularization of pulmonary vessels may also occur, resulting in severe and often intractable persistent pulmonary hypertension of the newborn (PPHN), the main cause of death.[2-5]

A recent study[6] has pointed out the survival benefits of initial stabilization and the use of ventilation strategies prior to definitive treatment, shifting the trend from immediate to delayed surgical repair. CDH can be repaired with either primary or patch closure; however, in many cases, it is unsuccessful because of serious abnormalities in the pulmonary architecture, altered vasoreactivity, and vascular remodeling, which occur antenatally.[7]

In recent years, the ability to diagnose CDH at the prenatal stage has notably improved, and due to the use of ultrasonography and/or magnetic resonance imaging, it is possible to document CDH at an early gestational age.[8] In most developed countries, about two thirds of CDH cases are diagnosed by routine antenatal ultrasonography in the second or third trimester. A prenatal diagnosis of CDH could prompt the transfer of the mother and fetus to a tertiary care center specialized in CDH cases.[9-12]

Therefore, invasive antenatal interventions have been proposed to promote *in utero* lung growth and decrease neonatal CDH mortality, with the most promising one being fetal endoscopic tracheal occlusion (FETO). Tracheal occlusion prevents the amniotic fluid outflow from the lungs into the amniotic cavity. Through mechanical action, it causes accelerated pulmonary growth, alveolarization and alveolar distension, decreasing the deleterious effects of pulmonary hypoplasia and PPHN. However, tracheal occlusion has adverse effects on type II cells and induces a major surfactant deficiency. In addition it is known that the balloon causes changes in the trachea, such as local inflammatory changes and epithelial defects.[13-15]

Sildenafil improves alveolar growth, preserve lung angiogenesis and decrease right ventricular hypertrophy in animal models of bronchopulmonary dysplasia.[15] Also, endothelin-1 (ET-1) promotes endothelial cell dysfunction, smooth muscle cell proliferation and remodeling. ET-1 binds to two receptor subtypes, endothelin receptors A and B; bosentan is a dual receptor antagonist.[16]

We hypothesized that modulation of cyclic guanosine monophosphate and endothelin pathways with sildenafil and bosentan prenatally administered respectively could attenuate the morphological pulmonary abnormalities in the vasculature and airways[17-20] in nitrofen-induced CDH. Nitrofen, an herbicide and teratogen that produced diaphragmatic defects in embryonic rats, has been extensively used because of the striking resemblance of the diaphragmatic, pulmonary and other associated defects to those in infants with CDH.[21,22]

Methods

All procedures and protocols were approved by the Institutional Animal Care and Use Committee of the Western Biomedical Research Center, Mexican Social Security Institute, Guadalajara, Jalisco, Mexico (NOM-062-ZOO-2001), and the experiments were done according to the institutional guidelines, which comply with those approved by International Institutes of Health in respect to the human treatment of research animals.

Animal model

Pregnant Wistar rats were gavage-fed with, at post-conception day (PCD) 9, 100 mg of herbicide nitrofen (2,4-dichlorophenyl 4-nitrophenyl ether, CAS No. 1836-75-5; Supelco®) dissolved in 1 mL of olive oil vehicle and mixed with a sonicator (VirSonic 100®) to induce CDH in the offsprings, except for the control group or negative group.

Study groups

Pregnant Wistar rats were randomly divided into five groups: group 1 (negative control) each rat was administered with 1 mL of olive oil on PCD 9 and 1 mL of 5% glucose solution during PCD 16 to 20. In groups 2-5, each pregnant rat was given 100 mg of nitrofen on PCD 9, reconstituted in 1 mL of olive oil, and also on PCD 16 to 20: group 2 (positive control) was given 1 mL of 5% glucose solution; group 3 each pregnant rat was given 100 mg of sildenafil per kg bodyweight and per dose, diluted in 1 mL of 5% glucose solution; group 4 received 30 mg of bosentan per kg bodyweight and per dose, diluted in 1 mL of 5% glucose solution; group 5 each pregnant rat was given 100 mg of sildenafil per kg and per dose and 30 mg of bosentan per kg and per dose, each diluted in 0.5 mL of 5% glucose solution, administered in separate doses. All doses were administered by gavage-fed with an orogastric cannula.

Lung tissue preparation and lung morphology

Pregnant rats required to complete 12 offsprings per group were used. On gestational day 21, after administration of xylazine and pentobarbital at 50 mg/kg body weight and per dose, cesarean delivery was

performed. The offspring was sacrificed and weighed on a portable digital scale (CS-200 Ohaus®). Through laparotomy using a binocular surgical microscope (Carl Zeiss® OPMI 1, surgical microscope, Germany), the diaphragmatic defect was identified in order to confirm CDH, and pulmonary hypoplasia was assessed too. Through sternotomy, the lung was dissected and weighed with a digital analytical balance (Explorer Ohaus®), recording the length and width of both lungs. The lung was placed in 10% buffered formalin and embedded in paraffin. Serial sections of the pulmonary parenchyma were taken with a microtome (Leica®, series 050131379, cat. 14050237993) and stained with hematoxylin and eosin, periodic acid Schiff and with Masson trichrome. The stained sections were analyzed with a microscope (Olympus® BX51) equipped with an eye piece micrometer and image analysis software (Image-pro® Plus, MediaCybernetics). The sections were examined by the pathologist who was unaware of what group each section was taken from. The followings were recorded: arterial wall thickness, in microns; arterial lumen diameter, in microns; pulmonary arterial-wall-lumen ratio; terminal bronchioles average; alveolarization assessed by means of the radial alveolar count[23,24] and the alveolar morphological characteristics through mean linear intercept.[25,26]

Statistical analysis

The SPSS (statistical package for the social sciences) version 15 was used. Quantitative data were taken as median [95% confidence interval (CI)]. Using the Kolmogorov-Smirnov test, the data's distribution was evaluated, which was significant for the great majority of the variables; therefore, they were analyzed using nonparametric inferential statistics. The comparisons were conducted through the Mann-Whitney *U* test for independent samples, between the negative control group *vs*. the positive control or nitrofen group, in order to demonstrate the experimental CDH model. Likewise, the positive control or nitrofen group was compared versus groups 3 (sildenafil), 4 (bosentan) and 5 (sildenafil and bosentan). For qualitative variables, the Fisher's exact test was conducted and, for those cases where the variable under study had more than two categories, the Chi-square test was used. To analyze the probability of interaction between different treatments, a regression model was carried out. A statistically significant value was determined when *P* was less than 0.05 with a 95% confidence level.

Results

The offspring with CDH showed hypoplasia of the left lung, determined by reduction of the lung weight-to-body weight ratio after exposure to nitrofen: 0.026 (95% CI, 0.024-0.028) in contrast to 0.039 (95% CI 0.035-0.042) (*P*=0.0001) in the negative control group. The size of the left lung was significantly smaller (*P*=0.0001) in the offsprings exposed to nitrofen than in those from the negative control group [4.50 (95% CI 3.96-5.12) mm long and 2.75 (95% CI 2.22-2.86) mm wide, versus 8.00 (95% CI 7.57-8.76) mm long and 3.00 (95% CI 2.96-2.86) mm wide (*P*=0.003)].

Likewise, vascular remodeling was observed as a greater thickness of the arterial wall was found in the positive or nitrofen group (5.0; 95% CI 4.1-4.9 μm) (Table 1). There was an increase in vascular muscularization in comparison to the negative control group (3.0; 95% CI 2.3-3.9 μm) (*P*=0.004).

In addition to a reduction in alveolarization, compromised bronchiolar branching is another morphological finding. In our study, a clear and significant reduction (*P*=0.001) was found in terminal bronchioles in the offsprings with CDH in the positive control or nitrofen group [8.7 (95% CI 7.2-9.4) μm] compared with the negative control group [11.0 (95% CI 10.0-12.5) μm] (Fig.).

In the study groups which received pharmacological interventions, the offsprings with CDH whose mothers were given sildenafil (group 3) showed a significant reduction (*P*=0.02) in the arterial wall thickness in microns. The offsprings with CDH and no pharmacological intervention showed a 5.0 (95% CI 4.1-4.9) μm thickness of the arterial wall, but those

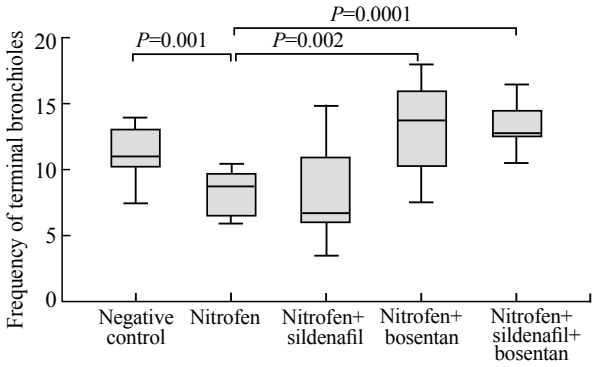

Fig. Median and 95% confidence interval for terminal bronchioles density in the negative control (*n*=12), positive control or nitrofen (*n*=12), nitrofen+sildenafil (*n*=12), nitrofen+bonsentan (*n*=12) and nitrofen+sildenafil+bosentan (*n*=12) groups.

Table 1. Arterial wall thickness (μm) from the different experimental groups

Arterial wall thickness (μm)	*n*	Median	95% CI
Group 1: Negative control	12	3.0	2.30-3.87
Group 2: Positive control or Nitrofen	12	5.0	4.07-4.93
Group 3: Nitrofen+sildenafil	12	3.0	2.77-3.73
Group 4: Nitrofen+bosentan	12	4.0	3.20-4.30
Group 5: Nitrofen+sildenafil+bosentan	12	3.0	2.35-4.48

CI: confidence interval.

Table 2. Arterial wall/lumen ratio of nitrofen without pharmacological intervention group versus nitrofen+sildenafil group and nitrofen+sildenafil+bosentan group

Arterial-wall-lumen ratio	Group 2: Nitrofen *n* (%)	Group 3: Nitrofen+sildenafil *n* (%)	Group 5: Nitrofen+sildenafil+bosentan *n* (%)
1:1	5 (41.7)	1 (8.3)	0 (0)
1:2	6 (50.0)	8 (66.7)	10 (83.3)
1:3	1 (8.3)	3 (25.0)	2 (16.7)
		P=0.03*	*P*=0.007*

*: comparisons between group 2 *vs.* 3 and 5. *n*: number of pups (*n*=12 per group).

whose mothers received sildenafil had a 3.0 (95% CI 2.8-3.7) μm thickness (Table 1).

As a result of the reduction in arterial wall thickness of the group that received sildenafil, the arterial-wall-lumen ratio improved significantly (*P*=0.03) (Table 2). In group 4 that received bosentan, there was a significant increase (*P*=0.002) in density of terminal bronchioles (Fig.). The density in the positive control or nitrofen group was 8.7 (95% CI 7.2-9.4) μm, whereas it was 13.7 (95% CI 10.7-15.2) μm in the group 4 or nitrofen+bosentan group.

The arterial wall thickness was reduced significantly (*P*=0.005) to 3.0 (95% CI 2.3-4.5) μm in the group 5 that received nitrofen+sildenafil+bosentan, whereas it was 5.0 (95% CI 4.1-4.9) μm in the positive control group (Table 1). Following the reduction of the arterial wall thickness, the arterial-wall-lumen ratio was improved in the group 5. There was a significant difference between the two groups (*P*=0.007; Table 2). Likewise, there was a significant increase (*P*=0.0001) in terminal bronchioles. In contrast to the positive control or nitrofen group the arterial wall thickness was 8.5 (95% CI 7.1-9.3) μm, but in the group 5 (nitrofen+sildenafil +bosentan) it was 14.0 (95% CI 12.5-15.4) μm (Fig.).

As to alveolarization, which was assessed by radial alveolar count and alveolar morphometric characteristics, through mean linear intercept, no significant differences were found between the groups that received only nitrofen (group 2 or positive control) versus nitrofen+pharmacological interventions (groups 3, 4 and 5).

In a regression model, interaction between the groups was dismissed with respect to arterial wall thickness and terminal bronchioles. In terms of sildenafil and bosentan treatments, which imply that the offspring with CDH who received sildenafil showed a reduction in arterial wall thickness. Likewise, with respect to the increase in terminal bronchioles, only bosentan was proved to be significantly effective.

Discussion

In the present study, the offsprings with CDH were shown to have left pulmonary hypoplasia measured by the lung-weight-to-body weight ratio, a crude indicator of lung hypoplasia which was lower in the offsprings who developed CDH, compared to the negative control group. The pulmonary morphological findings were consistent with previous reports.[27] Nitrofen-induced CDH in rats is a well-established and reliable model that recapitulates the pulmonary abnormalities described in human CDH, including lung hypoplasia and pulmonary vascular remodeling.[28]

Histopathological analysis of the specimens revealed thickening of the pulmonary vessel walls in the offsprings that were exposed to nitrofen and developed CDH, in contrast to those in the negative control group (Table 1). This increase in pulmonary vessel thickness has been considered a surrogate marker of vascular remodeling in infants with CDH who often present with refractory PPHN resistant to the pulmonary vasodilator inhaled nitric oxide.[20]

In addition to thickening of the arterial wall, decreased density of airspaces and bronchial branching were found in the CDH model, a *sine qua non* condition of pulmonary hypoplasia. Altogether, these pulmonary histopathological, morphologic, and structural changes constitute the basic characteristics of CDH and to some extent determine its physiopathogenesis.[29]

In groups 3, 4 and 5 which received sildenafil and/or bosentan, it was possible to attenuate pulmonary morphological, architectural and structural changes; especially those related to the abnormal development of the pulmonary vascular wall, as the characteristic of PPHN in patients with CDH and are the main cause of death of such patients.[30-32] Thus, group 3 which received sildenafil, a specific phosphodiesterase 5 inhibitor, showed a dilated pulmonary vasculature and antiproliferative effects on cells of the pulmonary artery.[33] There was a significant reduction in arterial wall thickness in microns, which helped to mitigate the reduction of the vessel's lumen, expressed as the arterial-wall-lumen ratio. Antenatal administration of sildenafil attenuated pulmonary vascular remodeling, which would lead us to expect less reactivity in pulmonary vascular tone at postnatal stages and probably, improved response to the therapeutic strategies such as inhaled nitric oxide.[34-38]

In the fetal lung development, the branching pattern remains one of the main characteristics of organic growth and development. Pulmonary branching determines the efficient functioning of the mechanism for gas exchange from the environment to the human body and vice versa. This development process begins

in uterus and evidence shows that it is considerably restricted in newborns with CDH. In humans, pulmonary bronchiolar branching is completed around week 27 of gestation during the canalicular stage and, in rats, it is completed around day 18 post conception.[31] In groups 4 and 5 which were given bosentan, an endothelin blocker, from PCD 16 to 20, the development of terminal or tertiary bronchioles was significantly improved. The bronchioles were coated with an epithelium capable of undertaking gas exchange.

Group 5 which received both sildenafil and bosentan also exhibited a significant reduction in the thickness of the vascular musculature. As a result, the arterial-wall-lumen ratio improved significantly. Another significant finding in group 5 was the increase of terminal or tertiary bronchioles, as was seen in the group 4.

In humans, the development of alveoli begins around 36 weeks of gestation and continues during the postnatal period. It begins with the emergence of alveolar septum, which are divided into primitive air sacs in the sacular stage of pulmonary development. Later, small spherical units are formed and transformed into the first alveoli. Throughout the first year of life, this gas exchange zone multiplies considerably and the diameter of the air spaces diminishes. The final remodeling of the alveolar structures takes place in adult age.[39] This fundamental event of pulmonary development, which is necessary to carry out the gas exchange, is interrupted by CDH.

In rats, alveolar development begins at the end of the gestational term, with an increase in the multiplication of alveolar sacs on postnatal days 3 and 8. The expansion of the alveoli continues at a sharp rate up to the first month of life but persists into adult age,[40] a situation which would partially explain why no positive changes were found in alveolarization by the proposed pharmacological interventions, as gestation was interrupted by cesarean on day 21 post conception and the offsprings were sacrificed precisely at the beginning of the development stage of alveoli. Thus, we consider that the methodology employed restricted an appropriate assessment of alveolarization. Although the alveolar evaluation techniques such as RAC and mean linear intercept have offered ample confidence in estimating the number and morphometric characteristics of such structures,[41] we consider that both the timing and manner of birth, as well as the fact that the offspring does not carry out any respiratory efforts, adversely influence the alveolar assessment. Unfortunately in the present study, it was not possible to evaluate the impact of the pharmacological interventions in terms of PPHN due to the fact that the offsprings were sacrificed at 21 days of gestation (they are normally born at the 22nd gestational day).

Though the survival rates of newborns with CDH have been improved by multidisciplinary treatments, there is yet a lot of research to be done in the field of antenatal interventions.[42]

In conclusion, in this rat model antenatal treatment with sildenafil attenuates pulmonary vascular remodeling in CDH, whereas antenatal administration of bosentan increases the density of terminal bronchioles and improves pulmonary architecture. The combination of pharmacological interventions, sildenafil and bosentan, promotes the formation of terminal respiratory airways and attenuates the increase in pulmonary arterial wall thickness in the experimental model used in this study.

Funding: This work was supported by grants from the Fondo de Fomento a la Investigación, Instituto Mexicano del Seguro Social (FOFOI-FIS, IMSS, 2006/1A/I/031-FIS/IMSS/PROT/205), and by Miami Children's Hospital & Sheridan Health Corporation.

Ethical approval: All procedures and protocols were approved by the Institutional Animal Care and Use Committee, and the experiments were done according to the Institutional guidelines, which comply with those approved by International Institutes of Health in respect to the humane treatment of research animals.

Competing interest: There are no conflicts of interest associated with this work.

Contributors: All authors listed here have participated substantially in the design, acquisition of data, analysis and interpretation of data, in addition to having reviewed the final draft and have approved this version for publication.

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Received October 22, 2013
Accepted after revision February 26, 2014