Hirosaki Med. J. 65：74—81，2014

ORIGINAL ARTICLE

A MORPHOMETRIC INVESTIGATION OF PULMONARY SYMPATHETIC INNERVATION IN NITROFEN-INDUCED CONGENITAL DIAPHRAGMATIC HERNIA RATS

Tamotsu Kobayashi[1)]，Michihiro Sugai[1)]，Fumiaki Mori[2)]，Koichi Wakabayashi[2)] and Kenichi Hakamada[1)]

Abstract Pulmonary hypoplasia influences prognosis in congenital diaphragmatic hernia (CDH). However, the abnormality of pulmonary innervation is not clearly understood. We immunohistochemically examined the lungs of CDH model rats, with special reference to sympathetic innervation. Pregnant rats were received nitrofen on gestational day 9 and the fetuses were recovered on gestational day 22. Control animals received only olive oil. Fetal lungs were dissected and the weight was measured. Whole mount sections at the level of main bronchus were immunostained using anti-tyrosine hydroxylase (TH) antibody as a sympathetic marker. The proportion of area of TH-immunoreactive nerve fibers relative to the total lung area was calculated. The mean lung weight of CDH affected side was significantly lower than that of ipsilateral side of controls. The percentage of TH-positive area of CDH affected side was significantly lower than that of unaffected side. The reduction of pulmonary sympathetic innervation may play an important role in respiratory morbidity in CDH.

Hirosaki Med. J. 65：74—81，2014

Key words: congenital diaphragmatic hernia; pulmonary hypoplasia; autonomic innervation.

原　著

Nitrofen 誘発先天性横隔膜ヘルニアラットにおける肺の交感神経支配に関する定量形態学的検討

小林　完[1)]　須貝道博[1)]　森　文秋[2)]　若林孝一[2)]　袴田健一[1)]

抄録　先天性横隔膜ヘルニア(CDH)において肺低形成は予後に大きく影響を与える．しかし，肺の神経支配異常に関しては不明な点が多い．我々はCDHモデルラットを作成し，肺の交感神経について定量的に検討した．妊娠9日目のラットにオリーブオイルに溶解したnitrofenを投与し妊娠22日目に胎仔を取り出した.対照群にはオリーブオイルのみを投与した.胎仔の肺を摘出し重量を測定した．主気管支レベルの全肺の組織切片を作成し，交感神経のマーカーであるtyrosine hydroxylase (TH)に対する抗体を用い免疫染色を行った．全肺面積に対するTH陽性神経線維の面積比率を測定した．CDH患側肺の平均重量は対照群の同側肺に比べ有意に減少していた．CDH患側肺のTH陽性面積の比率は健側肺に比べ有意に低下していた．肺の交感神経支配の減少はCDHにおける呼吸機能障害に重要な役割を果たす可能性が示唆された.

弘前医学　65：74—81，2014

キーワード：先天性横隔膜ヘルニア；肺低形成；自律神経支配.

[1)] Department of Pediatric Surgery, Hirosaki University Graduate School of Medicine
[2)] Department of Neuropathology, Institute of Brain Science, Hirosaki University Graduate School of Medicine
Correspondence: K. Hakamada
Received for publication, December 5, 2013
Accepted for publication, December 18, 2013

[1)] 弘前大学大学院医学研究科小児外科学講座
[2)] 弘前大学大学院医学研究科脳神経病理学講座
別刷請求先：袴田健一
平成25年12月5日受付
平成25年12月18日受理

Introduction

Congenital diaphragmatic hernia (CDH) is a relatively common birth defect occurring in lower than 1 of 2,500 live-born infants and remains the most life-threatening cause of severe respiratory failure in the term infants[1, 2]. The anatomic abnormalities in CDH include a defect in the diaphragm associated with persistence of abdominal viscera in the thoracic cavity, as well as developmental abnormalities of lungs, including pulmonary hypoplasia and pulmonary vascular abnormalities[3].

Despite remarkable progress in resuscitation and intensive care, the morbidity and mortality rates in CDH remain high due to severe pulmonary hypoplasia and persistent pulmonary hypertension resulting from maldevelopment of the pulmonary vascular bed. Postnatal therapies such as extracorporeal membrane oxygenation, inhaled nitric oxide and surfactant, and high-frequency ventilation have had limited impact on the prognosis and survivors of CDH commonly face considerable morbidity[4]. Thus, pulmonary hypoplasia is considered as one of the major determinant of respiratory morbidity in survivors of CDH. However, the pathogenesis of pulmonary hypoplasia in CDH is not clearly understood.

The effect of neural development on organogenesis is an emerging area of interest, and the specific role of embryonic pulmonary innervation in modulation of normal lung development remains to be explored. Previous studies have demonstrated that tracheal and broncho-pulmonary innervation is decreased in nitrofen-induced CDH model[5,6]. Several investigators have also reported that the pulmonary parasympathetic nerves are decreased in nitrofen-induced CDH model[3,5]. However, Lath et al.[3] have reported that nitrofen-treated embryonic lungs exhibited altered autonomic innervation, with a relative increase in sympathetic nerve staining. To characterize the extent of pulmonary sympathetic innervation, we immunohistochemically and morphometrically examined the hypoplastic lungs of nitrofen-induced CDH rats, using antibody against tyrosine hydroxylase (TH).

Materials and Methods

The experiment was performed in accordance with Guidelines for Animal Experimentation, Hirosaki University.

Animals

Pregnant Sprague-Dawley rats (day 6 of gestation) were purchased from CLEA Japan, Inc. (Tokyo, Japan). The day of sperm positivity in vaginal smears taken from the rats was regarded as the first day of gestation (G1). G23 was generally the day of delivery.

Administration procedure

The rats were used in the experiment at G9. Nitrofen (100 mg, Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) was dissolved in 1 ml olive oil, and administered orally with a stainless catheter to 4 pregnant rats (nitrofen group). Three control rats were received 1 ml of olive oil alone on the same day (control group).

Gross and histological examination

Under diethylether anesthesia, pregnant rats at G22 were laparotomized and the fetuses were removed from the uterus. Forty fetuses from nitrofen group and 41 fetuses from control group were fixed with 10% formalin for 48 h. In each fetus, the diaphragm was carefully inspected for the presence of CDH using a dissecting microscope and both lungs were removed for histological examination. Nitrofen group was divided into three categories by the presence or absence of CDH (right CDH, CDH existed on the right side; left CDH, CDH

existed on the left side; non-CDH, CDH was not occurred). Cases with bilateral CDH were excluded in the following study. The weight of right and left lungs was measured in each case.

For whole mounts, embryonic lungs were dehydrated using a graded ethanol series and embedded in paraffin. Serial 4-μm-thick sections at the level of main bronchus were obtained. The sections were stained with hematoxylin and eosin or subjected to immunohistochemical investigations using the avidin-biotin-peroxidase complex (ABC) method with a Vectastain ABC kit (Vector, Burlingame, CA, USA) as reported previously[7]. Mouse monoclonal anti-TH antibody (TH-16; Sigma, St. Louis, MO, USA; 1:1,000) was used as a marker of sympathetic nerves. The sections were pretreated in a microwave oven for 15 min in 10 mM citrate buffer (pH 6.0). Diaminobenzidine was used as the chromogen. The sections were counterstained with hematoxylin.

Morphometric analysis

Digital images of lung sections immunostained with anti-TH under a 40x objective lens were captured at the highest resolution (2776 x 2074 pixels) on a Macintosh personal computer (Apple Computer, Inc., Cupertino, CA, USA) with a Pro 600ES digital camera system (Pixera Co., Los Gatos, CA, USA). For quantification of TH-immunoreactive nerve fibers, we utilized Adobe Photoshop CS5 software (Adobe systems, San Jose, CA, USA) to convert color images to black and white. The proportion of the area of TH-immunoreactive nerve fibers relative to the total lung area was calculated using Image J software (National Institutes of Health) in each case.

Statistical analysis

All values were presented as mean + standard deviation. Statistical significance was evaluated using Student's *t*-test when comparing two conditions: difference in the mean weight or the percentage of TH-positive area between right and left lungs in each category. Non-parametric two-way ANOVA (Friedman's test) was used to test for the effect of the presence of CDH on the lung weight or the percentage of TH-positive area. Furthermore, post-hoc test (Fisher's Protected Least Significant Difference) was used to evaluate possible differences in the lung weight or the percentage of TH-positive area between control, right CDH, left CDH and non-CDH. Calculations were performed using Statcel software (OMS Publishing, Tokorozawa, Japan). Probability values less than 0.05 ($P < 0.05$) were considered significant.

Results

A total of 40 fetuses were removed from 4 nitrofen-exposed pregnant rats. CDH occurred in 32 fetuses (80.0%); right CDH (n = 17), left CDH (n = 9), bilateral CDH (n = 6) and non-CDH (n = 8) (Figure 1). Cases with bilateral CDH were excluded in the following study.

Table 1 shows the mean weight of right and left lungs in four categories (control, right CDH, left CDH and non-CDH). In all categories, the mean weight of right lung was significantly higher than that of left lung ($P < 0.01$). The non-parametric two-way ANOVA (Friedman's test) showed a significant effect of the presence of CDH on the lung weight ($P < 0.01$). The mean lung weight of CDH affected side was significantly lower than that of ipsilateral side of controls (right lung, $P < 0.01$; left lung, $P < 0.01$) (Figure 2a, b). The mean weight of right lung of right CDH was significantly lower than that of ipsilateral lung of non-CDH ($P < 0.01$) and left CDH ($P < 0.05$). The mean weight of left lung of right CDH was significantly lower than that of ipsilateral lung of controls ($P < 0.01$). There was no significant difference in the lung weight between controls and non-CDH.

TH-immunoreactive sympathetic nerve fibers

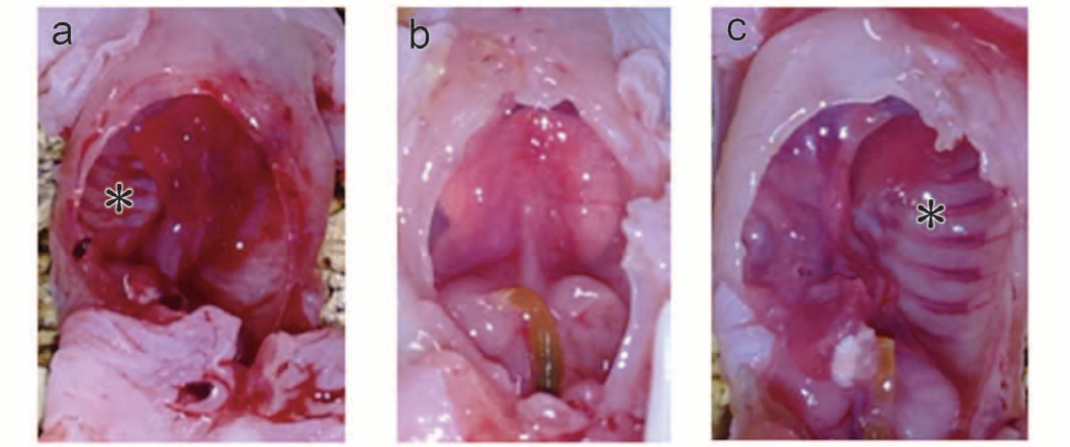

Figure 1 Macroscopic view of congenital diaphragmatic hernia (CDH) rats administered orally with nitrofen. **a**: Partial defect of the right diaphragm (asterisk). **b**: The presence of bilateral diaphragm. **c**: Partial defect of the left diaphragm (asterisk).

Table 1 Lung weight (mg) of control and nitrofen-induced congenital diaphragmatic hernia (CDH) rats

	Rt. lung	Lt. lung	*P* value
Control (n = 15)	93.1 ± 13.9	50.5 ± 9.21	< 0.01
Rt. CDH (n = 10)	39.3 ± 11.6	27.1 ± 10.4	< 0.01
Lt. CDH (n = 6)	70.7 ± 28.1	32.8 ± 16.0	< 0.01
Non-CDH (n = 6)	72.5 ± 16.3	40.0 ± 15.3	< 0.01

were found in peribronchiolar and perivascular regions in control and nitrofen groups (Figure 3). The TH staining pattern was less complex in CDH affected lungs compared with controls (Figure 3). Quantification of TH immunostaining revealed a significant decrease in the proportion of TH-positive area of CDH affected side compared with unaffected side (right CDH, $P < 0.05$; left CDH, $P < 0.01$) (Table 2). The non-parametric two-way ANOVA showed a significant effect of the presence of CDH on the percentage of TH-positive area ($P < 0.01$). The percentage of TH-positive area of right lung of right CDH was significantly lower than that of right lung of left CDH ($P < 0.01$) (Figure 4a). The percentage of TH-positive area of left lung of left CDH was significantly lower than that of left lung of right CDH ($P < 0.05$) (Figure 4b). The percentage of TH-positive area of CDH affected side was lower than that of ipsilateral lung of controls and non-CDH, but the difference

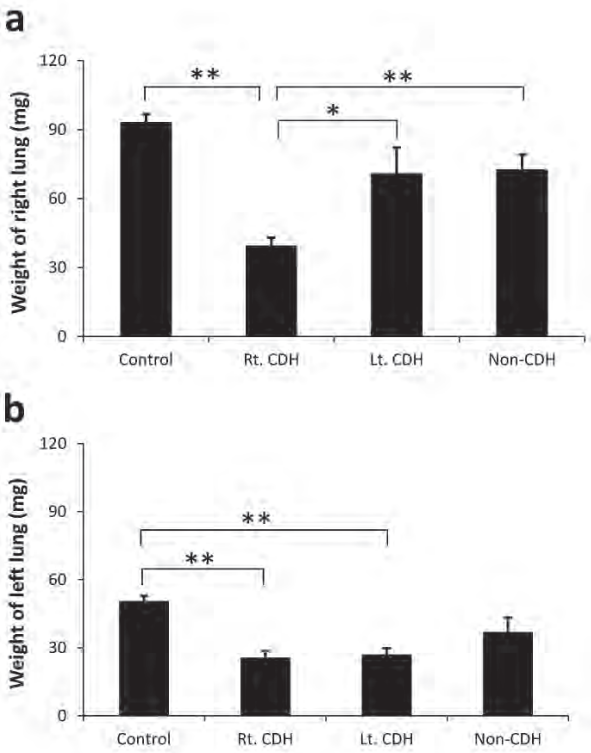

Figure 2 Comparison of the lung weight (**a**: right lung; **b**: left lung) in control and nitrofen-induced CDH rats. *$P < 0.05$, **$P < 0.01$.

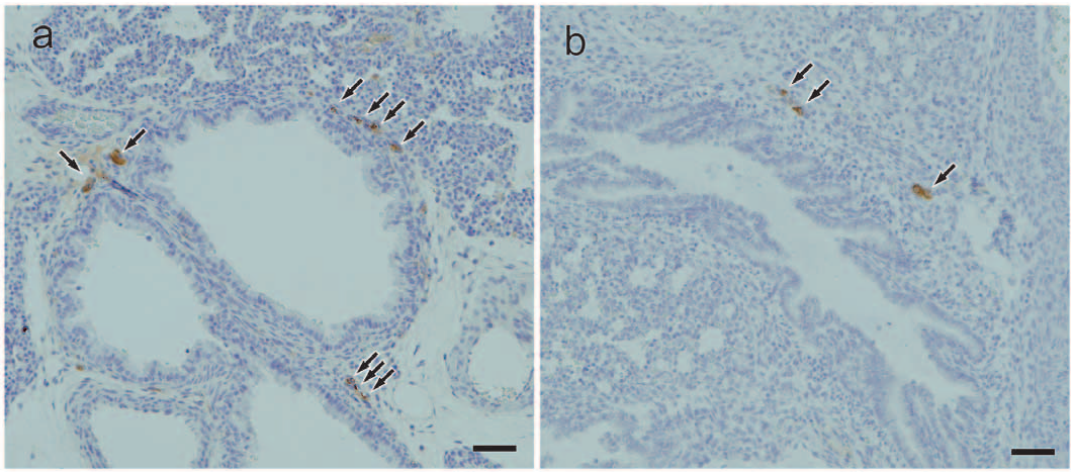

Figure 3 Light micrographs of right lungs of control (**a**) and right CDH rat (**b**). Tyrosine hydroxylase-immunoreactive sympathetic nerves (arrows) are decreased in nitrofen-induced CDH rat (**b**) compared with control (**a**). Bars = 100 μm.

Table 2 Tyrosine hydroxylase-positive area (% of total lung area)

	Rt. lung	Lt. lung	P value
Control (n = 7)	0.0862 ± 0.0123	0.0483 ± 0.0212	> 0.05
Rt. CDH (n = 4)	0.0164 ± 0.00124	0.186 ± 0.0720	< 0.05
Lt. CDH (n = 6)	0.142 ± 0.0254	0.00978 ± 0.00307	< 0.01
Non-CDH (n = 7)	0.0733 ± 0.0207	0.116 ± 0.0323	> 0.05

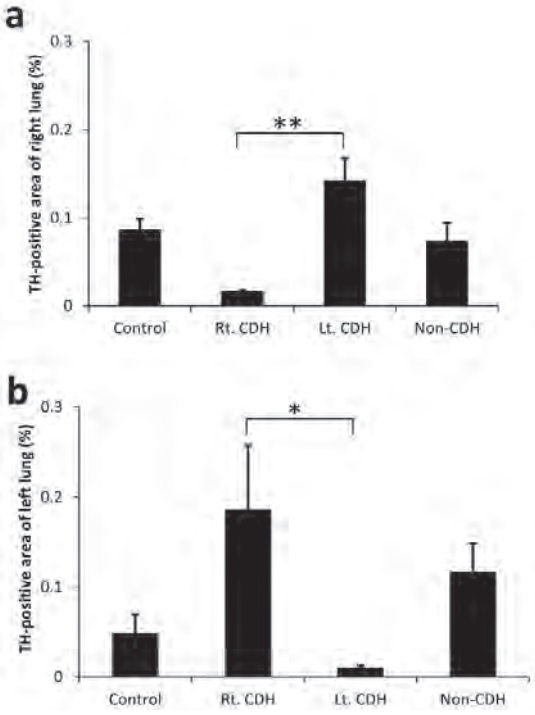

Figure 4 Comparison of the proportion of tyrosine hydroxylase (TH)-positive area relative to the total lung area (**a**: right lung; **b**: left lung) in control and nitrofen-induced CDH rats. $^{*}P < 0.05$, $^{**}P < 0.01$.

was not statistically significant. There was no significant difference in the percentage of TH-positive area between controls and non-CDH.

Discussion

CDH is a relatively common birth defect, resulting in significant mortality and long-term morbidity in survivors[8-10]. The lungs in infants born with CDH exhibit specific developmental defects, including varying degrees of lung hypoplasia and decreased pulmonary arterial branching, with increased muscularization of the intrapulmonary arteries[11, 12]. These abnormalities can lead to severe pulmonary hypertension and respiratory distress in the neonatal period. Pulmonary hypertension is a major factor contributing to poor outcomes in infants with CDH[13]. However, the underlying mechanism contributing to the development of lung hypoplasia, innervation abnormality and

pulmonary vascular defects in CDH remains undefined.

The CDH model rats induced by nitrofen have been widely used to investigate the pathogenesis of CDH and other associated malformations[14]. The pulmonary vascular abnormalities (decreased pulmonary arterial branching, increased muscularization of intrapulmonary arteries, and increased expression of α-smooth muscle actin) in this model are similar to those in human CDH[15]. Interestingly, nitrofen is thought to act through inhibition of retinoid signaling by inhibiting retinal dehydrogenase[16]. Retinoid signaling has been shown to influence the development of neural crest and peripheral nerves, suggesting that defective neural development may be a key early factor in the maldevelopment of lungs in CDH[17, 18].

In the experiments by Lath et al.[3], pregnant mice were received nitrofen on gestational day 8.5 and the fetuses were examined from embryonic day 12.5 (E12.5) to E16.5. About 70% of nitrofen-exposed embryos developed CDH. All nitrofen-treated embryonic lungs exhibited significant hypoplasia from the early stages of lung development onward, with decreased airway branching and vascular defects characteristic of CDH even before closure of the diaphragm. These findings suggest that the lung developmental defects may occur during the early stages of lung development, regardless of the presence of the diaphragmatic defect *per se*.

In the present study, we compared the lung weight to evaluate the lung hypoplasia in nitrofen-induced CDH rats. Leinwand et al.[19] demonstrated that the total lung weight of CDH model was significantly lower than that of controls at E18. In our experiment, the lung weight of CDH affected side was significantly lower than that of ipsilateral lung of controls. However, there was no significant difference in the weight of both lungs between controls and non-CDH. These data suggest that the herniation of abdominal viscera into the thoracic cavity may have more strong influence on the reduction of lung weight than the lung maldevelopment due to nitrofen-exposing. The reduction of lung weight in CDH may represent lower lung volume and hypoplastic pulmonary vessels and alveoli.

We further examined the lungs of nitrofen-exposed embryos using anti-TH antibody as a marker of sympathetic nerves. The percentage of TH-positive area of CDH affected side was significantly lower than that of unaffected side. The percentage of TH-positive area of right lung of right CDH was significantly lower than that of right lung of left CDH. The percentage of TH-positive area of left lung of left CDH was significantly lower than that of left lung of right CDH. In addition, there was no significant difference in the percentage of TH-positive area between controls and non-CDH. These findings suggest that the herniation of abdominal viscera into the thorax may have more strong influence on the immaturity of pulmonary autonomic innervation than nitrofen itself. Considering that autonomic innervation controls tracheobronchial mucosa, airway smooth muscles and blood vessels, immature pulmonary autonomic innervation may play an important role in respiratory morbidity in CDH.

Lath et al.[3] demonstrated that peripheral nerves immunolabeled with anti-protein gene product 9.5 (PGP9.5) (a pan-neuronal marker) were significantly decreased in nitrofen-treated mice lungs from E12.5 to E16.5 compared with controls. Furthermore, nitrofen-treated embryonic lungs exhibited altered autonomic innervation, with a relative increase in sympathetic nerve staining and a relative decrease in parasympathetic nerve staining compared with controls. These findings may imply that a primary defect of pulmonary

neural development results in less complex neural innervation and autonomic imbalance. By contrast, sympathetic nerves were significantly decreased in CDH affected side compared with unaffected side in our study. The discrepancy between our results and those of Lath et al.[3] may be explained by the difference of embryonic stage examined: we examined the lungs of rats at E22 and Lath et al. examined the lungs of mice from E12.5 to E16.5. Several investigators have shown that functional innervation of the embryonic lung begins in the early stages of lung development[20-22]. Furthermore, neurotransmitters and neuropeptides are expressed in the early stages of lung development[6, 23]. It is likely that altered autonomic innervation, with a relative increase in sympathetic nerves, is caused by nitrofen in the early stage of lung development and that subsequent diaphragmatic hernia further influences the autonomic innervation in the late stage of lung development.

In conclusion, we demonstrated the reduction of lung weight and pulmonary sympathetic innervation in nitrofen-induced CDH rat model. The maldevelopment of pulmonary autonomic innervation may play an important role in respiratory morbidity in CDH.

Acknowledgements

This work was supported in part by Hirosaki University Institutional Research Grant (K.W.). The authors wish to express their gratitude to M. Nakata for her technical assistance.

References

1) Stege G, Fenton A, Jaffray B. Nihilism in the 1990s: the true mortality of congenital diaphragmatic hernia. Pediatrics. 2003;112:532-5.

2) Torfs CP, Curry CJ, Bateson TF, Honoré LH. A population-based study of congenital diaphragmatic hernia. Teratology. 1992;46:555-65.

3) Lath NR, Galambos C, Rocha AB, Malek M, Gittes GK, Potoka DA. Defective pulmonary innervation and autonomic imbalance in congenital diaphragmatic hernia. Am J Physiol Lung Cell Mol Physiol. 2012;302:L390-8.

4) Montedonico S, Nakazawa N, Puri P. Congenital diaphragmatic hernia and retinoids: searching for an etiology. Pediatr Surg Int. 2008;24:755-61.

5) Pederiva F, Lopez RA, Martinez L, Tovar JA. Tracheal innervation is abnormal in rats with experimental congenital diaphragmatic hernia. J Pediatr Surg. 2009;44:1159-64.

6) Pederiva F, Lopez RA, Rodriguez JI, Martinez L, Tovar JA. Bronchopulmonary innervation defects in infants and rats with congenital diaphragmatic hernia. J Pediatr Surg. 2010;45:360-5.

7) Mori F, Nishie M, Kakita A, Yoshimoto M, Takahashi H, Wakabayashi K. Relationship among α-synuclein accumulation, dopamine synthesis, and neurodegeneration in Parkinson disease substantia nigra. J Neuropathol Exp Neurol. 2006; 65:808-15.

8) Boloker J, Bateman DA, Wung JT, Stolar CJ. Congenital diaphragmatic hernia in 120 infants treated consecutively with permissive hypercapnea/spontaneous respiration/elective repair. J Pediatr Surg. 2002;37:357-66.

9) Kays DW, Langham MR Jr, Ledbetter DJ, Talbert JL. Detrimental effects of standard medical therapy in congenital diaphragmatic hernia. Ann Surg. 1999;230:348-51.

10) Lally KP, Lally PA, Van Meurs KP, Bohn DJ, Davis CF, Rodgers B, Bhatia J, et al. Treatment evolution in high-risk congenital diaphragmatic hernia: ten years' experience with diaphragmatic agenesis. Ann Surg. 2006;244:505-13.

11) Gosche JR, Islam S, Boulanger SC. Congenital diaphragmatic hernia: searching for answers. Am J Surg. 2005;190:324-32.

12) Taira Y, Yamataka T, Miyazaki E, Puri P. Comparison of the pulmonary vasculature in newborns and stillborns with congenital diaphragmatic hernia. Pediatr Surg Int. 1998;14:30-5.

13) Dillon PW, Cilley RE, Mauger D, Zachary C, Meier

A. The relationship of pulmonary artery pressure and survival in congenital diaphragmatic hernia. J Pediatr Surg. 2004;39:307-12.

14) Gonçalves FL, de Oliveira FS, Schmidt AF, Pereira LA, Gallindo RM, Figueira RL, Sbragia L. Effect of nitrofen in the final stages of development of the diaphragm muscle in rats. Acta Cir Bras. 2013;28 (Suppl 1):13-8.

15) Coleman C, Zhao J, Gupta M, Buckley S, Tefft JD, Wuenschell CW, Minoo P, et al. Inhibition of vascular and epithelial differentiation in murine nitrofen-induced diaphragmatic hernia. Am J Physiol. 1998;274:L636-46.

16) Greer JJ, Babiuk RP, Thebaud B. Etiology of congenital diaphragmatic hernia: the retinoid hypothesis. Pediatr Res. 2003;53:726-30.

17) Maden M. Retinoic acid in the development, regeneration and maintenance of the nervous system. Nat Rev Neurosci. 2007;8:755-65.

18) Maschhoff KL, Baldwin HS. Molecular determinants of neural crest migration. Am J Med Genet. 2000;97:280-8.

19) Leinwand MJ, Tefft JD, Zhao J, Coleman C, Anderson KD, Warburton D. Nitrofen inhibition of pulmonary growth and development occurs in the early embryonic mouse. J Pediatr Surg. 2002;37:1263-8.

20) Burns AJ, Thapar N, Barlow AJ. Development of the neural crest-derived intrinsic innervation of the human lung. Am J Respir Cell Mol Biol. 2008;38: 269-75.

21) Sparrow MP, Weichselbaum M, McCray PB. Development of the innervation and airway smooth muscle in human fetal lung. Am J Respir Cell Mol Biol. 1999;20:550-60.

22) Tollet J, Everett AW, Sparrow MP. Development of neural tissue and airway smooth muscle in fetal mouse lung explants: a role for glial-derived neurotrophic factor in lung innervation. Am J Respir Cell Mol Biol. 2002;26:420-9.

23) Vaccaro R, Parisi Salvi E, Renda T. Early development of chick embryo respiratory nervous system: an immunohistochemical study. Anat Embryol (Berl). 2006;211:345-54.