DOI: 10.1002/pd.4789

ORIGINAL ARTICLE

Myocardial effects of fetal endoscopic tracheal occlusion in lambs with CDH

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ABSTRACT

Introduction Fetal endoscopic tracheal occlusion in congenital diaphragmatic hernia (CDH) may reduce pulmonary hypertension and ameliorate postnatal cardiac output. The effects of sustained early (ETO) and late (LTO) tracheal occlusion on left ventricular (LV) cells in the lamb model have not been described.

Materials and methods CDH was created in lambs at 70 days' gestation (term = 145 days). ETO (85 days) or LTO (105 days) was sustained till term. After cesarean section (140 days) fetuses were euthanized and hearts harvested. LV myocardial cells were studied by histological and immunofluorescence (TGF-beta 1, endothelin-1) assays in CDH, ETO, LTO, and the control group (two subjects per group). Small intramyocardial arteries were evaluated by traditional histology.

Results LV myocardial histology in CDH and LTO was similar. ETO-induced LV myocardial cell enlargement and increased endothelin-1 and TGF-beta 1 staining; a weaker immunofluorescence signal was observed in LTO compared with ETO. Myocardial vascular wall thickness was greater in CDH than in controls. ETO was associated with a vascular wall thickness within the range of controls.

Conclusion With only two fetuses in each group, only an explorative evaluation was possible. The time point at which TO is performed seems to have an effect on cardiac morphology. Functional studies as well as confirmation in clinical samples are mandatory.

Funding sources: None
Conflicts of interest: None declared

INTRODUCTION

The incidence of congenital diaphragmatic hernia (CDH) is about 1/3000 live births. Despite improvements in neonatal care, lung hypoplasia and pulmonary hypertension are the causes of the high mortality (approximately 40–50%).

Left-sided cardiac structures are underdeveloped in left diaphragmatic hernia.[1–3] The relationship between left heart dimensions and outcome has not been well characterized. When prenatally detected, a small left ventricle (LV) is considered by some as a poor prognostic factor,[4–6] since at delivery it would be less compliant and unable to maintain normal cardiac function.[7,8] However, it has not been defined whether prenatal indices of left heart size and function are associated with postnatal outcome in patients with CDH, or what degree of apparent left heart hypoplasia is compatible with postnatal survival and a biventricular circulation.[3] It remains unclear whether this is a primary event or anomaly secondary to direct compression exerted on the heart by the herniated intra-thoracic organs.[8–10] The structural immaturity of the heart in CDH has already been described in animal models, and the extracellular matrix alteration seems to contribute to cardiac maldevelopment.[11]

Fetal endoscopic tracheal occlusion (FETO) has been proposed as prenatal therapy to ameliorate the prognosis in CDH.[12–19] FETO, usually performed at 26 to 32 weeks of gestational age, has been shown to increase fetal lung growth.[20] Recently, FETO has been applied earlier, at 22–24 weeks, in extreme cases of severe lung hypoplasia with the purpose of improving the pulmonary response, both at the level of the airways and vessels.[21]

While benefits on pulmonary tissue have been reported and reversal of pulmonary hypoplasia has been documented, the mortality rate remains high.[18,19] The displacement in the cardiac axis and a smaller LV yet without demonstrable effects on LV function and conduction has been documented in CDH human fetuses. So far no adverse changes in the cardiac axis, LV size, or cardiac function were documented after fetal intervention.[22] So far, no structural changes in CDH myocardial tissue and its vessels before and after FETO have been reported in humans or in animal models.

In this pilot study the effects of sustained early (ETO) and late (LTO) tracheal occlusion on fetal myocardial tissue were analyzed in the CDH lamb model. These preliminary results are reported with the aim of adding data on the role of the left heart impairment in CDH.

MATERIALS AND METHODS

Animals and surgical technique

The experimental protocol was approved by the Local and National Animal Care and Ethics Committees and was conducted in accordance with Italian and European legislation (D.lgs. 116/92, European Directives 86/609/EE for the protection of animals used in scientific and experimental studies and 2010-63UE).

Twenty pregnant ewes (Ripollese breed), aged 18–24 months, were enrolled. All animals included in the study were considered healthy on physical examination. Animals were allowed to acclimatize for 7 days in group standard housing facilities, in temperature controlled rooms (15–25 °C). Before the experiments the sheep were fasted overnight, but given free access to water. An ultrasound study was performed to confirm the pregnancy and the gestational age. The gestation length in sheep varies from 142 to 152 days (average 147 days). The pregnant ewes were given general anesthesia prior to undergoing the interventions: induction of a left-sided CDH on gestational day 70, ETO at day 85, during the end of the canalicular period of lung development, or LTO at day 105, during the terminal saccular period. Cesarean section was performed at 140 days of gestation. Vital functions of the pregnant ewes were monitored continuously to prevent complications.

Based on the surgical procedure, the fetuses were divided into three groups: CDH (fetuses with induced CDH), ETO (fetuses with induced CDH and ETO), and LTO (fetuses with induced CDH and LTO). Consequently, five animals were assigned to each group. Because of the high fetal mortality at CDH induction and/or at TO, data at term were available only for 6 fetuses (two subjects per group). Six healthy twins of operated animals were not enrolled in this evaluation. The control group included two littermates in case of a twin pregnancy, not undergoing fetal surgery.

Anesthesia protocol

All surgical procedures were performed under general anesthesia. After premedication with an intramuscular (IM) injection of 5 mg/Kg ketamine and 0.2 mg/Kg midazolam (Dormicum®, Roche), intravenous (IV) access was established at the external jugular vein.

The pregnant ewes were pre-oxygenated using a facemask and induced with 5 mg/Kg IV propophol (Propofol®-Lipuro 1%, B. Braun Melsungen AG). Endotracheal intubation was performed, and general anesthesia was maintained with isoflurane 2% (Isoflo, Abbott laboratories Ltd) in 100% oxygen (1.5 L/min).

Esophageal intubation was performed to prevent ruminal bloat. A continuous infusion of Ringer lactate (B. Braun Melsungen AG) was administered at 10 mL/Kg/h during surgery. Intra-operative monitoring consisted of standard electrocardiography, pulse oximetry, non-invasive blood pressure, and capnography. Maternal body temperature was monitored by using a digital probe and maintained at 36–37 °C with a warming bed. All animals received a 75 μg transdermal fentanyl patch (Durogesic®, Janssen Pharmaceutics) immediately after surgery and Meloxicam 0.5 mg/kg IM/24 h for seven days for post-operative pain relief. For peri-operative infection prophylaxis, the animals received a single dose of 22 mg/Kg IV cephazolin (Kurgan®, Normon Laboratories) at the time of induction and 15 mg/Kg IM amoxicillin (Duphamox L.A., Fort Dodge) every 48 h, for eight days. Fetuses were anesthetized through the placenta, and additional anesthesia with fentanyl (10 μg/kg IM) and muscle relaxants pancuronium (0,3 mg/Kg IM) was administered.

Surgical technique

As a first step, a diaphragmatic hernia was surgically induced (day 70 of gestation). A midline laparotomy exposed the gravid uterus. The fetus was partially exteriorized from the uterus through a 4 cm hysterotomy. A left fetal thoracotomy was made, and part of the diaphragm was resected, and the fetal stomach was pulled into the thorax, which was closed in one layer. The fetus was returned to the uterus, 2 g of amoxicillin were added to the amniotic fluid, and the uterus wall and abdominal wall were closed.

For tracheal occlusion, again, the uterus was exteriorized through a midline laparotomy; the fetal mouth was located and partially exteriorized through a 2 cm hysterotomy. A detachable latex balloon (diameter 12 mm, length 28 mm, filled with maximum 2,5 mL, Goldbal 5®) was endoscopically placed into the trachea and inflated. The fetus was returned to the uterus; again, 2 g of amoxicillin were added to the amniotic fluid; and the uterus wall and abdominal wall were closed.

At day 140 of gestation (term = 145–150 days), lambs were delivered via cesarean section. Lambs were euthanized with a bolus of pentobarbital 200 mg/Kg IV. The harvested hearts were fixed in 10% buffered formalin (pH7) for 24–48 h before histological evaluation and immunofluorescence studies. Body weight of fetuses and lung weight were collected.

Histological evaluation and immunofluorescence staining

After fixation, the free wall of each LV was embedded in paraffin. The blocks were serially cut into 3 μm axial sections starting, just below the atrio-ventricular valves. After hematoxylin/eosin staining, histological analysis was performed. Endothelin-1 (ET-1) receptor A, TGF-β, and collagen I + smooth muscle actin antibodies (ABCAM, Cambridge, UK) were used for the

immunofluorescence evaluation. Analyses were performed under a light microscope (Olympus® Bx61). The sections were digitally photographed, and fluorescence signals were analyzed using Image J software. The nucleus/cytoplasmatic ratio[23] was obtained using a semi quantitative analytical micrometer scale.

A qualitative definition[24–26] of the arteries was used, which was based on the histological evaluation of small intramyocardial arteries identified as vessels having a poor muscular layer, moderate lumen, and without an evident elastic layer. The myocardial arterial muscular wall thickness was obtained using the image J software, and the values were calculated after normalization for internal diameter for statistical analysis.

Statistical analysis

Histological variables were reported as the mean and standard deviation; 200 measurements were made per sample/group, by the same pathologist (RB) blinded to the treatment groups. Normal distribution was checked with the Shapiro–Wilk test. All descriptive statistical analyses were performed using the SPSS statistical package (SPSS, Chicago).

RESULTS

Histology

The myocardial histological pattern was preserved in the CDH and LTO groups, as compared with controls. In these groups, myocytes had central nuclei, and cellular bodies were connected at the extremities by fibers, each divided by a thin connective layer. In the endomysium, a rich number of capillaries, deriving from the coronaries, were present (Figure 1A, B, and D).

In ETO, the LV showed increased myocyte size and a decreased nucleus/cytoplasmatic ratio. The cytoplasm was larger, and a poor, almost immature, and unstructured contractile component of the cells was observed (Figure 1C). The myocardial arteries' appearance in the CDH, LTO, and ETO groups showed no evident changes in terms of numbers compared with the control group; however, differences were noted in arterial muscular wall thickness (Figure 2). In CDH fetuses, the arterial walls were thicker than the control group ($0.89 \pm 0.07\,\mu m$ *vs* $0.84 \pm 0.09\,\mu m$). ETO was associated with a decrease in thickness ($0.72 \pm 0.12\,\mu m$) compared with controls. The LTO group showed no differences ($0.82 \pm 0.10\,\mu m$) with controls.

Immunofluorescence results

TGF-β1 expression was not observed in the control or CDH group (Supplementary Figure 1). While a weak signal was noted in the LTO group, the ETO group showed marked TGF-β1 expression (Figure 3C and D). ET-1 expression was detected in all specimens; fluorescence signals were observed in correspondence with the subendocardium, at the highly specialized cardiac cells, such as Purkinje fibers (Supplementary Figure 2). Only, in the ETO and LTO groups, ET-1 was highlighted in myocardial tissue. While a slight signal was detected in the LTO group, a stronger signal was observed in the ETO group. In these specimens the

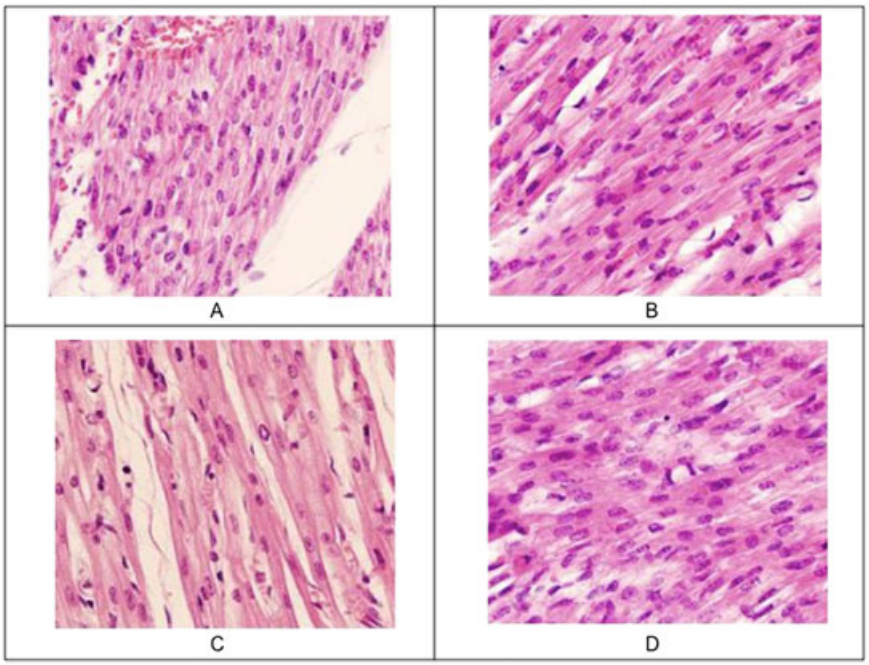

Figure 1 Left ventricles, hematoxylin-eosin staining in the control group (A), CDH (B), ETO (C), LTO (D), 40×. In A, B, D, myocytes show the typical cardiac pattern: central nuclei, and cellular bodies are connected at the extremities by fibers, each divided by a thin connective layer, at the endomisium. A dense trophic network of blood capillaries deriving from the coronaries is present in the endomisium. Capillaries are directly connected with muscular elements, thus providing nutritive elements. In C, the heart maintains its structural integrity and a dense net of capillaries is still present. However, cells show increased size with a decreased nucleus/cytoplasmatic ratio, swollen cytoplasm, and immature and poorly structured cellular contractile component (Figure 1C)

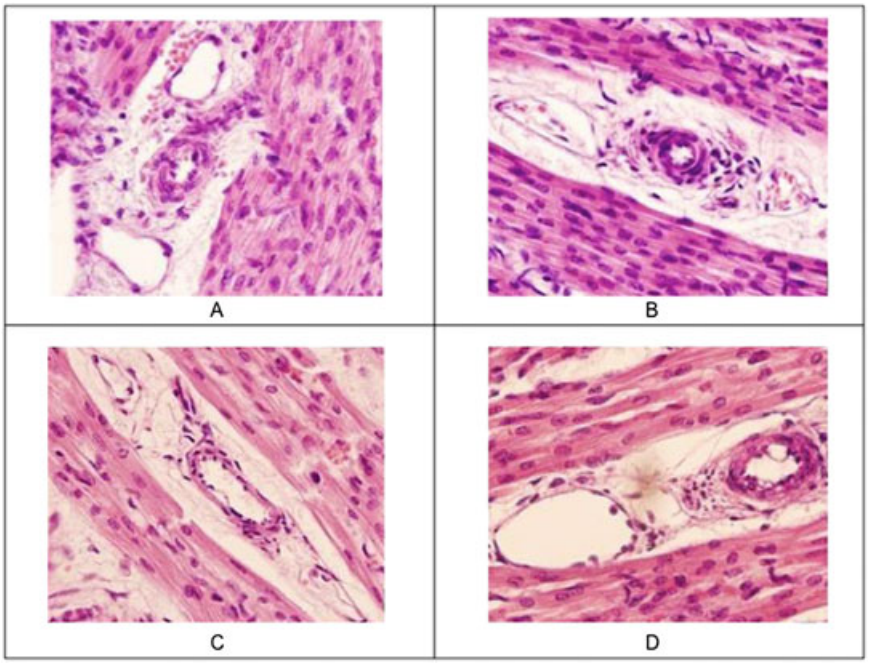

Figure 2 Left ventricles–arterial walls. Hematoxylin–eosin staining in the control group (A), CDH (B), ETO (C), LTO (D), 40–. Small caliber arteries show a variation in thickness. Compared with the control, group B arteries have increased muscular thickness with reduction of the inner area. Panel C shows a marked reduction of the muscular component and a significant increase of inner vascular area with respect to controls. In Panel D, wall arterial thickness is not different from panel A

gradient extended from the endocardium to myocardium (confirmed at a 40× magnification: Supporting Information Figure 2). Analysis with smooth muscle actin antibodies confirmed the differences in wall thickness previously reported in the groups.

Macroscopic data

The average of lung wieght/body weight ratios was smaller in CDH than in controls (0.010 and 0.035, respectively) and higher in ETO than in LTO (0.086 and 0.075, respectively).

DISCUSSION

Isolated CDH is still associated with high rates of mortality and morbidity. Although predicting neonatal outcome remains a challenge, FETO may improve neonatal survival as occlusion of the fetal trachea prevents egress of lung fluid, hence accelerates lung growth and pulmonary vascularization. When TO is induced between 26 and 32 weeks, it promotes fetal pulmonary growth over four weeks.[12–15,18,19] Fetuses with more severe pulmonary hypoplasia show a diminished pulmonary response and worse postnatal prognosis.[21] Therefore, performing FETO earlier has been suggested in cases with extremely severe CDH.

Preliminary results on the benefits of early FETO have been recently described.[21] TO performed between 22 and 24 weeks' gestation induces a significant growth in fetal pulmonary size and remarkably improves pulmonary vascularization. The benefits seem to persist over a longer period of almost 6 weeks following the procedure.[21] Despite these improvements, the mortality rate in neonates with CDH continues to remain high because of persistent pulmonary hypertension,[18,19] and left-sided cardiac underdevelopment represents a poor prognostic factor in some studies.[3–6]

Cardiac evaluation is an important part of the investigation in a fetus with CDH. Prenatal sonographic detection of underdeveloped left-sided heart is considered by some as a poor prognostic factor.[4–6] In CDH fetuses, the heart is displaced by the physical presence of the abdominal contents in the chest and flow dynamics within the heart are altered. Secondary diminution of ventricular growth, particularly of the left heart, has been documented in human CDH fetuses.[8] The present histologic data are in line with experimental data in the nitrofen-induced CDH rat model.[11,27]

The combination of a smaller ventricular size and normal function suggests a redistribution of cardiac output toward the right ventricle when sustained by an adequate lung volume. Smaller lungs are associated with impaired pulmonary blood flow, resulting in augmented shunting through the ductus arteriosus and higher impedance to the LV outflow. Moreover, large lungs, and hence a higher pulmonary blood flow, lead to improved preload of the LV.

Following FETO, a theoretical concern is that the acute increase in intrathoracic pressure, because of the expanded balloon and/or the rapid increase in lung volume, might influence cardiac function.[22] As shown by Rocha,[2] the increased fetal lung size after FETO is associated with enhanced fetal pulmonary blood flow, resulting in an increased *in utero* LV preload, which may ultimately lead to larger left cardiac structures. This adaptation probably reduces shunting through the ductus arteriosus.

As cardiac growth continues throughout *in utero* life,[28] the pulmonary venous return becomes an important determinant

of LV growth in the late third trimester.[29] The increased fetal lung size and enhanced pulmonary blood flow after TO may induce different responses and LV growth in ETO and LTO. According to the literature, fetal cardiac response to 'stressors' after TO seems to be gestational age-related, likely related to milestones in fetal cardiac development, as the myocardium prepares for *ex utero* life.[30]

In our study, the cardiac histological pattern was preserved in CDH and LTO fetuses. Surprisingly, in ETO, increases in myocyte cell size and a stronger expression of ET-1 were noted. Data from our study may be interpreted as indicative of functional adaptation of the cardiovascular system. ETO could contribute to high pulmonary pressure, causing right-to-left shunting of blood across the ductus arteriosus and foramen ovale with a possible subsequent myocardial, intra-myocardial, and interstitial stasis. Myocardial enlargement at this age of gestation (85 days) may be a compensatory response of myocardial tissue upon increased mechanical load. That condition may influence growth, maturation, and function of the myocytes.[29–33] ETO may thus induce structural changes in the left ventricular chambers to compensate for a LV that is unable to satisfy the increased demands or only at the expense of normal function at this age of gestation.

The strong ET-1 expression supports the hypothesis that ETO increases the amount of contractile myofibers.

ET-1 is a vasoactive peptide which is produced by a variety of cells, including cardiac myocytes. In addition to its vasoconstrictive effects, it has a potent mitogenic effect. ET-1 has been well documented in different studies *in vivo* and *in vitro* as one of the crucial factors in the development of cardiomyocyte hypertrophy.[32,34]

This study also showed higher TGF-β1 expression in ETO than in LTO specimens. TGF-β1 is a locally generated cytokine, and it may play a central role in protecting the heart during the hypertrophic response by helping to restore normal function of the affected myocardium.[34] Altered expression and biological activity of TGF-β1 in hypertrophic cardiomyocytes have been described, and it is likely that ET-1 regulates TGF-β1 expression in cardiac cells.[32,35–37] However, the relation and interaction between TGF-β1 and ET-1 remains unclear.[32] The different TGF-β1 and ET-1 expression in LTO supports the idea that LTO has a protective role on the cardiomyocyte.

A difference in cardiac arterial wall thickness between groups was observed in this preliminary study. In CDH, the muscular compartment was thicker than in the control group. In the LTO group, wall thickness was not different with respect to controls, while a reduction in arterial wall thickness in the ETO group was documented. Our data suggest that the adverse effects of CDH in fetuses are probably not restricted to the lung yet also involve LV myocardial cells and vessels.

We recognize that there were several limitations to this study. First, our data are mainly qualitative. In addition, observations in only two fetuses per group limits a proper statistical analysis. A quantitative analysis would help provide a better understanding of the cardiac impairment. We did not provide any confirmation at the protein or RNA level, nor were there cardiac functional or structural evaluations conducted *in vivo*. Also, clinical studies would need to confirm these observations. Finally, our assessment focused only on cardiac structures.

CONCLUSIONS

This study provides early data concerning left cardiac ventricular responses to TO procedures. Our preliminary results suggest that CDH and sustained TO have effects on myocardial cells and cardiac vascularization. The time point at which TO is performed seems to have an effect on cardiac morphology. Functional and clinical studies are mandatory to confirm the results.

ACKNOWLEDGEMENT

We wish to acknowledge the 'Associazione Bambini con la CCAM' for supporting research in the University of Pavia, Pediatric Surgery Unit.

WHAT'S ALREADY KNOWN ABOUT THIS TOPIC?

- Fetal tracheal occlusion (TO) may ameliorate the prognosis in CDH.
- Fetal TO improves lung size and pulmonary vascularization.
- Cardiac structural changes and effects after fetal TO are poorly described.
- Late TO promotes fetal pulmonary growth over four weeks.
- Early TO improves the pulmonary response in extremely severe CDH over six weeks.

WHAT DOES THIS STUDY ADD?

- Early TO stimulates myocardial cell enlargement.
- Early TO induces cellular edema and inhibits growth.
- Early TO seems to reduce the vascular arterial wall thickness.
- Late TO seems to protect cardiomyocytes from cell edema.

REFERENCES

1. Crawford DC, Drake DP, Kwaitkowski D, *et al.* Prenatal diagnosis of reversible cardiac hypoplasia associated with congenital diaphragmatic hernia: implications for postnatal management. J Clin Ultrasound 1986;14:718–21.
2. Rocha LA, Byrne FA, Keller RL, *et al.* Left heart structures in human neonates with congenital diaphragmatic hernia and the effect of fetal endoscopic tracheal occlusion. Fetal Diagn Ther 2014;35:36–43.
3. Vogel M, McElhinney DB, Marcus E, *et al.* Significance and outcome of left heart hypoplasia in fetal congenital diaphragmatic hernia. Ultrasound Obstet Gynecol 2010;35:310–17.
4. Baumgart S, Paul JJ, Huhta JC, *et al.* Cardiac malposition, redistribution of fetal cardiac output, and left heart hypoplasia reduce survival in neonates with congenital diaphragmatic hernia requiring extracorporeal membrane oxygenation. J Pediatr 1998;133:57–62.
5. Thebaud B, Azancot A, de Lagausie P, *et al.* Congenital diaphragmatic hernia: antenatal prognostic factors. Does cardiac ventricular disproportion in utero predict outcome and pulmonary hypoplasia? Intensive Care Med 1997;23:1062–69.
6. Schwartz SM, Vermillion RP, Hirschl RB. Evaluation of left ventricular mass in children with left-sided congenital diaphrag- matic hernia. J Pediatr 1994;125:447–51.

7. Aggarwal S, Stockmann P, Klein MD, Natarajan G. Echocardiographic measures of ventricular function and pulmonary artery size: prognostic markers of congenital diaphragmatic hernia? J Perinatol 2011;31:561–66.
8. Karamanoukian HL, Wilcox DT, Glick PL. The "missing link" in congenital diaphragmatic hernia. J Pediatr Surg 1994;29:954–55.
9. Allan LD, Irish MS, Glick PL. The fetal heart in diaphragmatic hernia. Clin Perinatol 1996;23:795–812.
10. Keijzer R, Liu J, Deimling J, *et al.* Dual-hit hypothesis explains pulmonary hypoplasia in the nitrofen model of congenital diaphragmatic hernia. Am J Pathol 2000;156:1299–306.
11. Guarino N, Shima H, Puri P. Structural immaturity of the heart in congenital diaphragmatic hernia in rats. J Pediatr Surg 2001;36:770–73.
12. Badillo A, Gingalewski C. Congenital diaphragmatic hernia: treatment and outcomes. Semin Perinatol 2014;38:92–96.
13. Haroon J, Chamberlain RS. An evidence-based review of the current treatment of congenital diaphragmatic hernia. Clin Pediatr (Phila) 2013;52:115–24.
14. Leeuwen L, Fitzgerald DA. Congenital diaphragmatic hernia. J Paediatr Child Health 2014 Feb 17. DOI:10.1111/jpc.12508.
15. Ritgen J, Kohl T, Enzensberger C, *et al.* Prenatal diagnosis of and therapy for congenital diaphragmatic hernia. Z Geburtshilfe Neonatol 2014;218:6–17.
16. Deprest JAM, Veerle A, Evrard K, *et al.* Tracheal side effects of endoscopic balloon tracheal occlusion in the fetal lamb model. Eur J Obstet Gynecol Reprod Biol 2000;92:119–26.
17. Chiu PP. New insights into congenital diaphragmatic hernia - a surgeon's introduction to CDH animal models. Front Pediatr 2014;2:36.
18. Ruano R, Yoshisaki CT, da Silva MM, *et al.* A randomized controlled trial of fetal endoscopic tracheal occlusion versus postnatal management of severe isolated congenital diaphragmatic hernia. Ultrasound Obstet Gynecol 2012;39:20–27.
19. 19. Jani JC, Nicolaides KH, Gratacós E, *et al.* Severe diaphragmatic hernia treated by fetal endoscopic tracheal occlusion. Ultrasound Obstet Gynecol 2009;34:304–10.
20. Deprest J, Gratacos E, Nicolaides KH. Fetoscopic tracheal occlusion (FETO) for severe congenital diaphragmatic hernia: evolution of a technique and preliminary results. Ultrasound Obstet Gynecol 2004;24:121–26.
21. Ruano R, Peiro JL, da Silva MM, *et al.* Early fetoscopic tracheal occlusion for extremely severe pulmonary hypoplasia in isolated congenital diaphragmatic hernia: preliminary results. Ultrasound Obstet Gynecol 2013;42:70–76.
22. Van Mieghem T, Gucciardo L, Doné E, *et al.* Left ventricular cardiac function in fetuses with congenital diaphragmatic hernia and the effect of fetal endoscopic tracheal occlusion. Ultrasound Obstet Gynecol 2009;34:424–29.
23. Swanson JA, Lee M, Knapp PE. Cellular dimensions affecting the nucleocytoplasmic volume ratio. J Cell Biol 1991;115:941-48.
24. Amann K, Gharehbaghi H, Stephan S, Mall G. Hypertrophy and hyperplasia of smooth muscle cells of small intramyocardial arteries in spontaneously hypertensive rats. Hypertension 1995;25:124–31.
25. Engelson ET, Schmidt-Schönbein GW, Zweifach BW. The microvasculature in skeletal muscle, II: arteriolar network anatomy in normotensive and spontaneously hypertensive rats. Microvasc Res 1986;31:356–74.
26. Weiss HR, Conway RS. Morphometric study of the total and perfused arteriolar capillary network of the rabbit left ventricle. Cardiovasc Res 1985;19:343–54.
27. Correia-Pinto J, Baptista MJ, Pedrosa C, *et al.* Fetal heart development in the nitrofen-induced CDH rat model: the role of mechanical and non mechanical factors. J Pediatr Surg 2003;38:1444–51.
28. Rasanen J, Debbs RH, Huhta JC. Echocardiography in intrauterine growth restriction. Clin Obstet Gynecol 1997;40:796–803.
29. Jonker SS, Zhang L, Louey S, *et al.* Myocyte enlargement, differentiation, and proliferation kinetics in the fetal sheep heart. J Appl Physiol 2007;102:1130–42.
30. Chattergoon NN, Louey S, Stork PJ, *et al.* Unexpected maturation of PI3K and MAPK-ERK signaling in fetal ovine cardiomyocytes. Am J Physiol Heart Circ Physiol 2014;307:H1216–25.
31. Germanakis I, Matsui H, Gardiner HE. Myocardial strain abnormalities in fetal congenital heart disease assessed by speckle tracking echocardiography. Fetal Diagn Ther 2012;32:123–30.
32. Shimojo N, Jesmin S, Zaedi S, *et al.* Eicosapentaenoic acid prevents endothelin-1-induced cardiomyocyte hypertrophy in vitro through the suppression of TGF-beta 1 and phosphorylated JNK. Am J Physiol Heart Circ Physiol 2006;291:H835–45.
33. Richey PA, Brown SP. Pathological vs. physiological left ventricular hypertrophy: a review. J Sports Sci 1998;16:129–41.
34. van Wamel AJ, Ruwhof C, van der Valk-Kokshoom LE, *et al.* The role of angiotensin II, endothelin-1 and transforming growth factor-beta as autocrine/paracrine mediators of stretch-induced cardiomyocyte hypertrophy. Mol Cell Biochem 2001;218:113–24.
35. Brand Tand Schneider MD. The TGF-β superfamily in myocardium: ligands, receptors, transduction, and function. J Mol Cell Cardiol 1995;27:5–18.
36. Gandhi CR, Kuddus RH, Uemura T, Rao AS. Endothelin stimulates transforming growth factor-β1 and collagen synthesis in stellate cells from control but not cirrhotic rat liver. Eur J Pharmacol 2000;406:311–18.
37. Harada M, Itoh H, Nakagawa O, *et al.* Significance of ventricular myocytes and non myocytes interaction during cardiocyte hypertrophy: evidence for endothelin-1 as a paracrine hypertrophic factor from cardiac non myocytes. Circulation 1997;96:3737–44.

SUPPORTING INFORMATION

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