

## ORIGINAL ARTICLES

## PRENATAL TRACHEAL LIGATION OR INTRA-AMNIOTIC ADMINISTRATION OF SURFACTANT OR DEXAMETHASONE PREVENTS SOME STRUCTURAL CHANGES IN THE PULMONARY ARTERIES OF SURGICALLY CREATED DIAPHRAGMATIC HERNIA IN RABBITS

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RHCFAP/3061

RODRIGUES CJ et al. - Prenatal tracheal ligation or intra-amniotic administration of surfactant or dexamethasone prevents some structural changes in the pulmonary arteries of surgically created diaphragmatic hernia in rabbits. *Rev. Hosp. Clín. Fac. Med. S. Paulo* 57(1):1-8, 2002.

**PURPOSE:** Characterization of the structural changes occurring in the pulmonary arteries resulting from surgically produced congenital diaphragmatic hernia in rabbits, with particular emphasis on the preventive effects of prenatal tracheal ligation or administration of intra-amniotic dexamethasone or surfactant.

**METHODS:** Twenty rabbit fetuses underwent surgical creation of a left-sided congenital diaphragmatic hernia on the 24th or 25th gestational day. They were divided according to the following procedures: congenital diaphragmatic hernia (n = 5), congenital diaphragmatic hernia plus tracheal ligation (n = 5), congenital diaphragmatic hernia plus intra-amniotic administration of dexamethasone 0.4 mg (n = 5) or surfactant (Curosurf 40 mg, n = 5). On gestational day 30, all the fetuses were delivered by caesarean section and killed. A control group consisted of five nonoperated fetuses. Histochemical analysis of medial thickness, cell nuclei density, and elastic fiber density of pulmonary arterial walls was performed.

**RESULTS:** Arteries with an external diameter > 100 µm have a decreased medial thickness, lower cell nuclei density, and greater elastic fiber density when compared with arteries with external diameter ≤ 100 µm. Congenital diaphragmatic hernia promoted a significant decrease in medial thickness and an increase in cell nuclei density in artery walls with external diameter > 100 µm. Prenatal treatments with tracheal ligation or intra-amniotic administration of dexamethasone or surfactant prevented these changes. In arteries with external diameter ≤ 100 µm, congenital diaphragmatic hernia promoted a significant increase in medial thickness and in cell nuclei density and a decrease in elastic fiber density. The prenatal treatments with tracheal ligation or intra-amniotic administration of dexamethasone or surfactant prevented these changes, although no effect was observed in elastic fiber density in the congenital diaphragmatic hernia plus dexamethasone group.

**CONCLUSIONS:** Congenital diaphragmatic hernia promoted different structural changes for large or small arteries. The prenatal intra-amniotic administration of dexamethasone or surfactant had positive effects on the lung structural changes promoted by congenital diaphragmatic hernia, and these effects were comparable to the changes induced by tracheal ligation.

**DESCRIPTORS:** Congenital diaphragmatic hernia. Persistent pulmonary hypertension. Pulmonary hypoplasia. Surfactant. Glucocorticoid.

Patients with congenital diaphragmatic hernia (CDH) have a high mortality rate, which has been attributed to pulmonary hypoplasia, pulmonary hypertension, and biochemical and structural immaturity of the lungs<sup>1-4</sup>. We have previously shown that fetal tra-

cheal ligation (TL) reverses pulmonary hypoplasia in a rabbit model of CDH

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by accelerating fetal alveolar growth. In the same series of experiments, we also demonstrated that prenatal intra-amniotic administration of surfactant or corticosteroids partially reverses the pulmonary hypoplasia of fetuses with CDH<sup>5</sup>.

Clinical and animal studies have shown that lungs with CDH are less compliant than normal lungs<sup>6,7</sup>. This low compliance is attributable to a higher surface tension caused by surfactant deficiency and abnormal stiffness of lung parenchyma, which may be caused by an increased concentration of collagen and decreased density of elastic fibers<sup>5,8</sup>. Hedrick et al.<sup>9</sup> demonstrated that prenatal glucocorticoid therapy improves pulmonary maturity parameters in rodent and sheep models of CDH. Taira et al.<sup>10</sup> reported that the administration of glucocorticoids has no effect on pulmonary tropoelastin and procollagen expression in lungs with CDH. However, in a previous study of lung glycogen content, elastic fiber density, and collagen content, we verified that the effects of prenatal intra-amniotic administration of surfactant or dexamethasone are comparable to changes induced by TL; therefore, these drug therapies may be substitutes for TL<sup>8</sup>.

The pulmonary vascular abnormalities that have been implicated in pulmonary hypertension in CDH are a decrease in the number of pulmonary arteries per unit lung volume, an increase in the medial thickness, and primarily the muscularization of small arteries<sup>11</sup>. Furthermore, some authors have recently reported that in addition to the changes in the medial thickness of small pulmonary arteries, increased adventitial thickness is also a consistent feature. These structural changes may result from an increase in intramural pressure<sup>12-14</sup>.

The purpose of the current study was to investigate the structural changes in the pulmonary arteries of surgically created diaphragmatic hernia in rabbits and the preventive effects of prenatal TL or intra-amniotic administration of dexamethasone or surfactant on these alterations.

## MATERIALS AND METHODS

### Experimental Animals

Twenty-five fetuses from 25 preg-

nant New Zealand rabbits were distributed into 5 groups. For the experimental groups, 20 fetuses underwent surgical creation of a left-sided CDH on the 24th or 25th gestational day as previously described<sup>5,8,15</sup> (the normal full gestation time is 31 days). They were divided according to the following procedures: CDH (n = 5), CDH plus TL (n = 5), CDH plus intra-amniotic administration of a single dose of dexamethasone (0.4 mg, n = 5) and CDH plus intra-amniotic administration of surfactant (40 mg of Curosurf, Pharmalab-Chiesi, São Paulo, Brazil, n = 5). After the surgical manipulations, each one received 400 mg of cephalothin intravenously and 5 mg of Depo-Provera<sup>®</sup> (NV, Upjohn, Belgium) intramuscularly.

On gestational day 30, all the fetuses were delivered by cesarean section and killed by intraperitoneal injection of thiobarbiturate. A control group consisted of 5 nonoperated fetuses.

### Tissue

Lung tissue specimens were obtained from the central region of the lower lobe of the left lung of each animal. The lungs were previously perfused through the trachea with buffered formalin solution (6%) at a pressure of 20 cm of H<sub>2</sub>O for 2 hours and immersed in the same formalin solution. The lung samples were embedded in historesin embedding medium (Leica) and sectioned at a setting of 1  $\mu$ m on a microtome (Leica RM 2035). The slides were stained for 10 minutes in a Meyer hematoxylin stain and for 1 hour in a resorcin-fuchsin (Weigert) stain for the nuclei and elastic fibers respectively.

### Morphometric Assessment of Pulmonary Arteries

To determine whether the characteristic structural changes were size-related, arteries were grouped according to Brenner<sup>16</sup>: (i) external diameter (ED)

> 100  $\mu$ m (the muscular arteries or pre-acinar arteries that accompany conducting airways); (ii) ED  $\leq$  100  $\mu$ m (the arterioles or intra-acinar arterioles associated with the respiratory bronchi and saccules).

Morphometric measurements were performed with an optical electronic image analysis system (Kontron 300, UK). A blinded investigator then studied lung sections from 5 fetuses in each of the 5 groups. The transversely sectioned pulmonary arteries present in the tissue were examined to measure the following parameters: external diameter (ED), the diameter between the external elastic lamina; internal diameter (ID), the diameter between the internal elastic lamina; elastic fiber density in the arterial wall (EF), expressed as a fraction of the area; and cell nuclei density in the arterial wall (CD), expressed as cell nuclei/ $\mu$ m<sup>2</sup>. The medial wall thickness (MT) was calculated as half the difference between the mean ED and ID, divided by ED, according to Best and Heath<sup>17</sup>. Therefore, MT was expressed as a percentage of the external diameter of the vessel to nullify the effects of vasodilatation or vasoconstriction on the measurements, as previously discussed<sup>18</sup>. Large arteries were measured on the monitor from the microscope image with x20 or x40 objectives, and the smaller ones were measured with a x100 objective.

### Statistical Analysis

The morphometric values were calculated from the groups and expressed as mean  $\pm$  standard deviation (SD), for each variable. All comparisons were performed using the analysis of variance (ANOVA) for parametric data or the ANOVA on Ranks and Dunn's method for multiple comparison procedure for non-parametric data. Analyses were performed with the SigmaStat 2.0 software package (Jandel Scientific). Significant results were obtained for  $P < .05$ .

## RESULTS

In all groups, pulmonary arteries with  $ED > 100 \mu\text{m}$  exhibited an evident internal elastic laminae with some others exhibiting elastic laminae in the medial wall (Fig. 1). Arteries with  $ED \leq 100 \mu\text{m}$  exhibited evident internal elastic laminae and some elastic fibers in the medial wall that may or may not constitute an elastic lamina. The small ones showed some elastic fibers in their medial wall (Fig. 2). Between these elastic laminae or fibers, we could see the nuclei of the smooth muscle cells arranged in concentric layers (Fig. 3).

The data from the morphometric analyses of pulmonary arteries with  $ED > 100 \mu\text{m}$  and  $ED \leq 100 \mu\text{m}$  are expressed in tables 1 and 2 and in figs. 4 to 6.

In all groups, the comparisons of the parameters for arteries with  $ED > 100 \mu\text{m}$  and arteries with  $ED \leq 100 \mu\text{m}$  showed significant differences ( $P < .05$ ). The muscular arteries ( $ED > 100 \mu\text{m}$ ) revealed lower MT and lower CD, but greater EF ( $P < .05$ ).

The comparison of the parameters of the arteries with  $ED > 100 \mu\text{m}$  between control and experimental groups showed that the CDH promoted a significant decrease ( $P < .05$ ) in MT. Considering the number of cell nuclei per square micrometer, we found that CDH promoted an increase in the cell density, in comparison to control animals. This change may be due to a decrease of the medial surface area of these arteries in the CDH lungs. The prenatal treatments with TL or intra-amniotic administration of dexamethasone or surfactant prevented these changes.

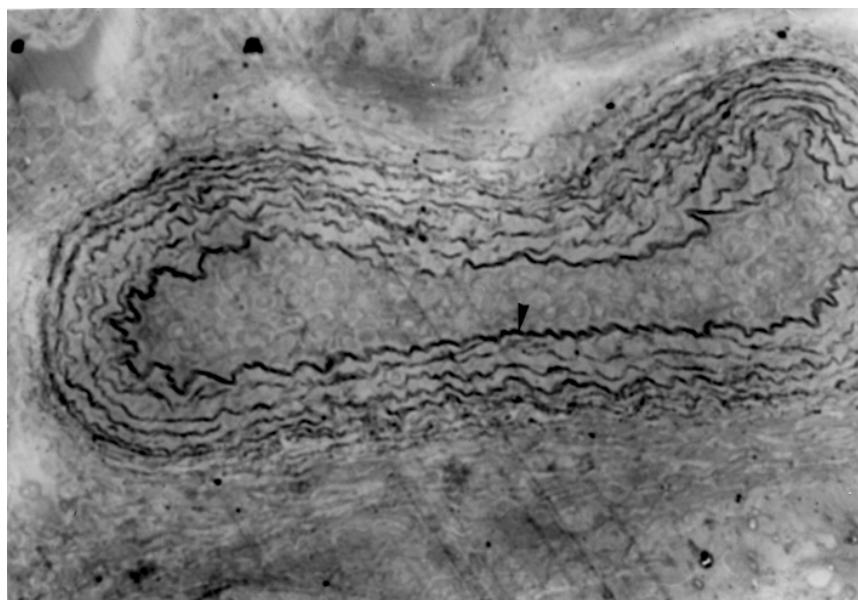
There was no difference between the control and experimental groups in EF in the walls of the pulmonary arteries with  $ED > 100 \mu\text{m}$ , although a non-significant decrease was observed in the CDH group.

The comparison between control

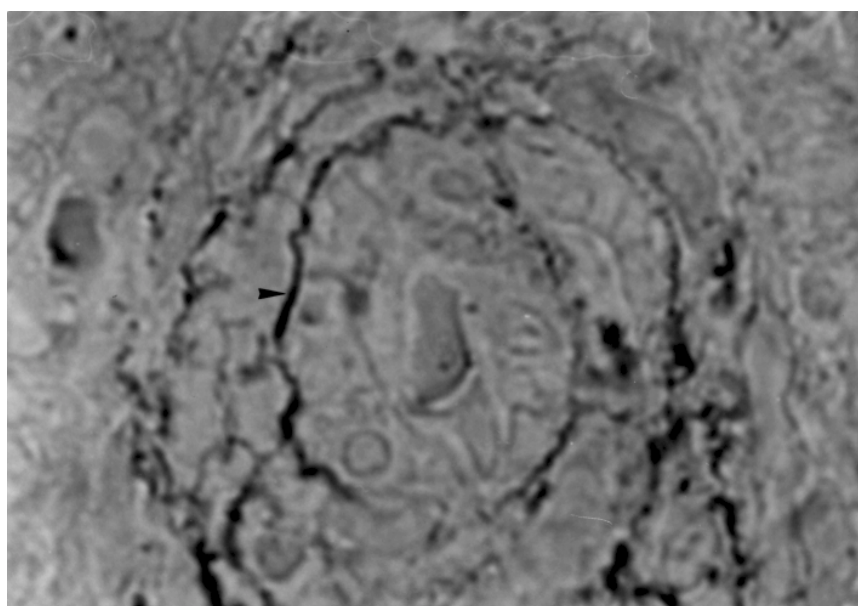
and experimental groups showed that CDH promoted a significant increase ( $P < .05$ ) in MT of the arteries with  $ED \leq 100 \mu\text{m}$ . We found that CDH promoted an increase in CD in comparison to that found in the control animals. This change may provide an explanation for the increased MT of these vessels. The prenatal treatments with TL or intra-amniotic administration of

surfactant or dexamethasone prevented these changes.

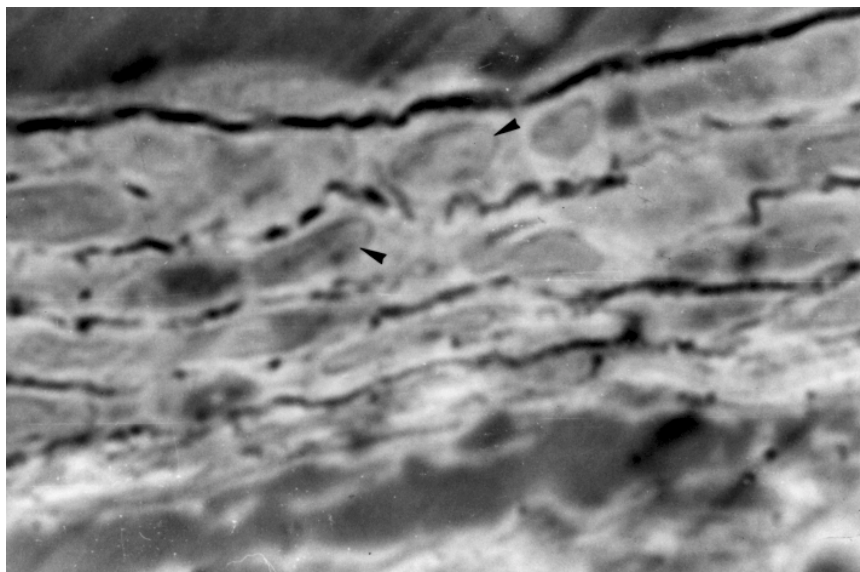
CDH induced a decrease in EF in arteries with  $ED \leq 100 \mu\text{m}$ . There was no production of elastic fibers with the increased number of cells. The prenatal treatments with TL or administration of surfactant prevented this change, although no effect was observed in the CDH plus dexamethasone group.



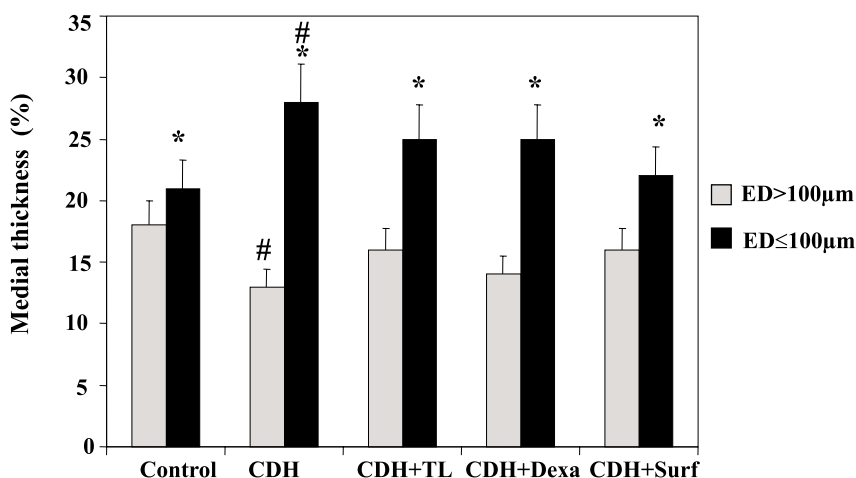
**Figure 1** - Micrograph of a pulmonary artery with  $ED > 100 \mu\text{m}$  showing an internal elastic laminae (arrowhead) with some other elastic laminae in the medial wall (Weigert-hematoxylin, x400).



**Figure 2** - Micrograph of a small pulmonary artery ( $ED \leq 100 \mu\text{m}$ ) showing some elastic fibers (arrowhead) in the medial wall (Weigert-hematoxylin, x2000).



**Figure 3** - Micrograph of medial wall of a pulmonary artery showing nuclei profiles (arrowheads) of the smooth muscle cells arranged in concentric layers between the elastic laminae (Weigert-hematoxylin, x2000).



**Figure 4** - Medial thickness (MT) of pulmonary arteries. (\*)  $P < .05$  - The comparisons between arteries with external diameter (ED) > 100 µm and ED ≤ 100 µm demonstrate that the muscular arteries (ED > 100 µm) have a decreased MT; (#)  $P < .05$  - relative to the control group. All the therapeutic procedures prevented the changes induced by congenital diaphragmatic hernia. The type of error bar (SD) should be noted.

**Table 1** - Values of morphometric data of arteries with external diameter > 100 µm.

	Control	Congenital diaphragmatic hernia	Congenital diaphragmatic hernia + Tracheal ligation	Congenital diaphragmatic hernia+Dexa	Congenital diaphragmatic hernia+Surf
External diameter(mean±SD)	243.4±160.13	181.5±65.41	170.7±65.58	213.4±78.22	183.9±75.45
Internal diameter(mean±SD)	155.3±98.46	136.3±54.75	117.1±47.15	156.2±63.67	127.7±58.59

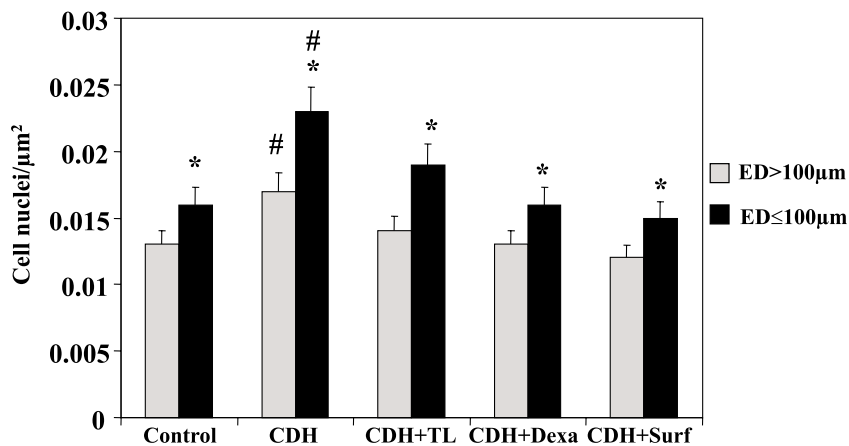
**Table 2** - Values of morphometric data of arteries with external diameter ≤ 100 µm.

	Control	Congenital diaphragmatic hernia	Congenital diaphragmatic hernia + Tracheal ligation	Congenital diaphragmatic hernia+Dexa	Congenital diaphragmatic hernia+Surf
External diameter(mean±SD)	64.6±18.93	44.5±20.66	52.7±19.61	51.1±18.04	57.1±21.77
Internal diameter(mean±SD)	37.6±11.76	22.8±19.21	27.7±16.88	27.5±15.71	32.5±18.65

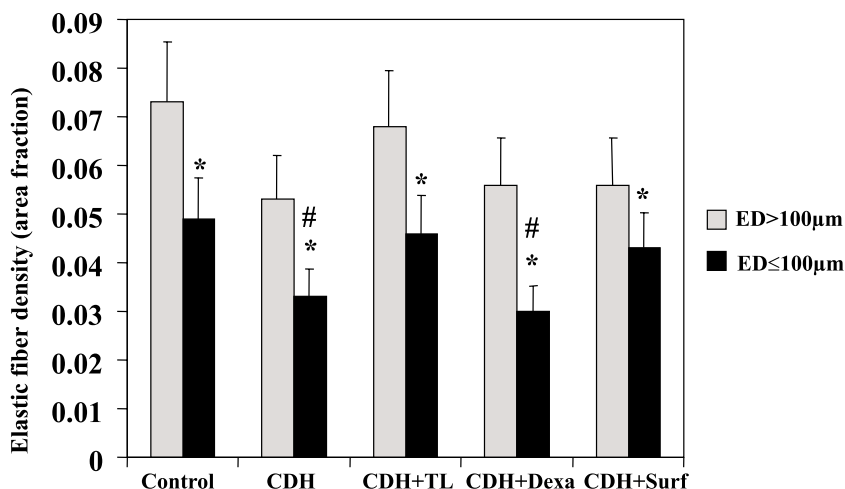
**DISCUSSION**

**Experimental model:** We chose the rabbit model of CDH because it was previously standardized in our laboratory and shown to produce pulmonary hypoplasia<sup>8,15</sup>. In the current study, we aimed to study whether prenatal therapeutic procedures could prevent the structural changes in the pulmonary arteries induced by surgically created CDH. To date, the only method that effectively treats or prevents all the changes of the herniated viscera on the developing lung in CDH fetus is tracheal occlusion, an invasive procedure. In 2 previous investigations, we proposed a new noninvasive therapeutic strategy, which consisted of prenatal intra-amniotic administration of high doses of swine surfactant (40 mg) or dexamethasone (0.4 mg), and we have proved that these treatments have positive effects on the immature lung of the fetal rabbit with CDH<sup>5,8</sup>, although we could not explain the mechanisms by which the intra-amniotic administered drugs produce their effects.

We chose the intra-amniotic route for administering the surfactant substance in the prenatal period to prevent pulmonary hypoplasia, since most investigations have used surfactant therapy prophylactically, i.e., before the onset of ventilation<sup>19</sup>. It has been suggested that when exogenous surfactant is considered for the newborn



**Figure 5** - Density of cell nuclei (CD) in the pulmonary arterial wall. (\*)  $P < .05$  – The comparisons between arteries with external diameter (ED) > 100 µm and ED ≤ 100 µm demonstrate that the muscular arteries (ED > 100 µm) have fewer cell nuclei per square micrometer; (#)  $P < .05$  – relative to the control group. All the therapeutic procedures prevented the changes induced by CDH.



**Figure 6** - Elastic fiber density (EF) (fraction of the pulmonary arterial wall area). (\*)  $P < .05$  – The comparison between arteries with external diameter (ED) > 100 µm and ED ≤ 100 µm; (#)  $P < .05$  – relative to the control group. There was no difference between the control and experimental groups in the arteries with ED > 100 µm. The therapeutic procedures, TL, and prenatal intra-amniotic surfactant administration prevented the change induced by CDH in the arteries with ED ≤ 100 µm.

with CDH, it should be administered as early as possible, preferably before the infant's first breath<sup>20</sup>. Concerning the route of antenatal administration of glucocorticoids, we also chose the intra-amniotic one because we had previously noted that the intramuscular injection of dexamethasone into the pregnant does had a significant labor-inducing effect, causing a high incidence of spontaneous abortion.

The respiratory movements of the fetus with inflow of the amniotic fluid

begin early in pregnancy, and in addition to being a normal function of the fetus, play an important role in the development of the lungs<sup>21</sup>. Although many previous studies suggest that the fetal lungs produce a fluid that flows constantly from the lungs to the amniotic cavity<sup>22,23</sup>, there is some evidence that drugs infused into the amniotic cavity may be aspirated by the fetus. In experiments of transamniotic fetal feeding using a rabbit model and lipid emulsion as the nutrient, it was observed that the

fetal alveoli were filled with lipid globules, which proved that amniotic fluid is chronically aspirated by the fetus<sup>24</sup>. In addition, in a previous similar investigation, we infused a mixture of porcine surfactant with microparticles of coal into the amniotic cavities of fetuses with CDH and of nonoperated littermates, and we noted that in all fetuses, the coal was massively aspirated into the lungs and trachea<sup>8</sup>.

It has been shown that CDH promotes alveolar hypoplasia, decreases the total size of the pulmonary vascular bed and the number of vessels per unit area of lung, and increases the muscularization of the arterial tree<sup>11,25-27</sup>. Levin<sup>28</sup> demonstrated that there is increased medial width and smooth muscle in resistance vessels in all lungs of patients with left-sided CDH and alveolar hypoplasia. Recently, Shehata et al.<sup>12</sup> showed that raised levels of vascular endothelial growth factor occur in the medial smooth muscle cells of large and small pulmonary arteries in patients with CDH.

In the present study, we demonstrated morphometric changes of the components of pulmonary arteries in rabbits with CDH, and we verified a reversal of these changes with prenatal fetal treatments, utilizing TL or intra-amniotic administration of dexamethasone or surfactant. Emphasis was given here on the quantitative morphological differences between large and small arteries in control and experimental animals.

**Morphometric measurements – medial wall thickness (MT):** In previous experiments, some authors reported that the hypoplasia of both large and capillary pulmonary vessels is related to a muscularization of pulmonary arterial vessels in animals with CDH. They reported that fetal TL is capable of reversing these effects by accelerating both large vessel growth and capillary growth based on total large vessel area and total capillary number<sup>1,28</sup>. The TL treat-

ment also appears to accelerate the lung maturation process<sup>19</sup>.

We also demonstrated that in the muscular pulmonary arteries with ED > 100  $\mu\text{m}$ , CDH promoted a decreased MT, probably as a result of a vasodilatation phenomenon, similar to what occurs in patients with atrial septal defect and other congenital heart diseases characterized by the presence of high pulmonary blood flow over a prolonged period prior to the onset of pulmonary hypertension. A dilatation occurs in pulmonary vessels with a thinning of the media as an expression of the compensation to severe pulmonary resistance<sup>29</sup>.

**Elastic fiber density and cell nuclei density in the pulmonary arterial wall:** By definition, elastic arteries are those with one or more elastic laminae between internal and external elastic laminae, whereas the muscular arteries have only the internal and external elastic laminae<sup>16,30</sup>. In our study, the pulmonary arteries with ED > 100  $\mu\text{m}$  were characterized as muscular arteries and exhibited one or more additional elastic laminae between the internal and external elastic laminae. In contrast, the smaller arteries with ED  $\leq$  100  $\mu\text{m}$  had only a crescent of smooth muscle, and the internal elastic laminae disappeared, with some elastic fibers being observed between the muscle cells. This phenomenon may be caused by a reduced capacity for response to the physiological actions of contraction or dilatation, since we have demonstrated that these small vessels have a greater density of cell nuclei than do the muscular arteries with ED > 100  $\mu\text{m}$ . We suggest that the large arteries control the blood flow, while the small ones are responsible for the resistance of the flow.

The walls of the arteries with ED  $\leq$  100  $\mu\text{m}$  are thicker and have more cells per unit area than the muscular arteries with ED > 100  $\mu\text{m}$ , and this finding agrees with those of previous studies<sup>1,25,28</sup>. The small arteries also have a lower tensile strength and thus can stretch farther to accommodate the higher interior pressure. The presence of abnormally thick-walled pulmonary arteries in stillborns with CDH suggests that the intrapulmonary arteries in CDH may become excessively muscularized during fetal life, becoming unable to adapt normally at birth<sup>31</sup>.

A previous investigation reported that some prenatal treatments improve these structural changes in the pulmonary arteries promoted by CDH. Lipsett et al.<sup>32</sup> demonstrated that tracheal occlusion performed in the lamb model of CDH stimulates lung development and restores lung structure and maturity towards normal levels by gestational term. There was also an increased volume density of parenchyma, airspace, and gas-exchange surface. Okoye et al.<sup>11</sup> studied the effect of prenatal glucocorticoids on pulmonary vascular muscularization in experimental CDH. They verified an increased MT ratio of pre-acinar and intra-acinar arteries in CDH and demonstrated that maternal administration of dexamethasone inhibits this medial hypertrophy. The same observation was made in rats with nitrofen-induced CDH<sup>33</sup>. However, Shehata et al.<sup>12</sup> showed that treatment with extracorporeal membrane oxygenation (ECMO) did not improve this increase in arterial medial thickness in newborns with CDH. The ECMO-treated newborns with CDH showed a thinner arterial adventitia than did patients with CDH who did not receive this treatment.

We observed that there is an increase in cell density and a decrease in the density of elastic fibers in small pulmonary arteries of animals with CDH. The prenatal treatments with TL or administration of surfactant or dexamethasone are capable of preventing the structural changes in arteries of all sizes, except that the administration of dexamethasone does not prevent any loss of elastic fibers in small pulmonary arteries. This last change may result in a less compliant arterial wall better suited to resisting the higher intra-arterial pressure. Yamataka and Puri reported similar results<sup>13,14</sup>, which showed an active synthesis of collagen in the media and adventitia of pulmonary arteries in patients with CDH complicated by persistent pulmonary hypertension.

In conclusion, the present study demonstrates that animals with CDH have different structural changes in large and small pulmonary arteries, confirming their diverse physiological function. Furthermore, this is the third study showing that prenatal intra-amniotic administration of dexamethasone or surfactant has positive effects on the lung structural changes promoted by CDH, and that these effects are comparable to the changes induced by TL. Further studies in lambs must be undertaken to confirm these results before embarking in a clinical trial in humans.

#### FINANCIAL SUPPORT

Supported in part by grants from FAPESP 94/6056-5, 94/6055-9, 95/07397-3 and Braun Laboratories, São Paulo-SP, Brazil.

## RESUMO

RHCFAF/3061

RODRIGUES CJ e col. - Ligadura de traquéia no período pré-natal ou administração intra-amniótica de surfactante ou dexametasona evitam algumas alterações estruturais nas artérias pulmonares de fetos de coelho com hérnia diafragmática congênita produzida com cirurgia. **Rev. Hosp. Clín. Fac. Med. S. Paulo** 57(1):1-8, 2002.

**OBJETIVO:** Caracterização das alterações estruturais que ocorrem nas artérias pulmonares de fetos de coelho com hérnia diafragmática congênita produzida com cirurgia, com destaque especial aos efeitos preventivos da ligadura de traquéia ou administração intra-amniótica de dexametasona ou surfactante.

**MÉTODOS:** Vinte fetos de coelho foram submetidos a cirurgia para produção de hérnia diafragmática no 24º ou 25º dia de gestação. Os animais foram divididos de acordo com os procedimentos: hérnia diafragmática congênita (n = 5), hérnia diafragmática congê-

nita com ligadura de traquéia (n = 5), hérnia diafragmática congênita com administração intra-amniótica de 0,4mg de dexametasona (n = 5) ou surfactante (40mg de Curosurf, n = 5). No 30º dia de gestação, todos os fetos foram retirados por cesariana e mortos. O grupo controle consistiu de 5 fetos normais não operados. Foram realizadas análises histomorfométricas da parede das artérias pulmonares: espessura da camada média, contagem do número de núcleos celulares e densidade de fibras elásticas.

**RESULTADOS:** As artérias com diâmetro externo > 100 µm apresentaram diminuição da espessura da camada média, menor número de núcleos celulares por micrômetro quadrado e maior densidade de fibras elásticas em comparação com as artérias com diâmetro externo diâmetro externo ≤ 100 µm. A hérnia diafragmática congênita provocou significativa redução na espessura da camada média e aumento do número de núcleos nas artérias com diâmetro externo > 100 µm. Os méto-

dos terapêuticos, ligadura de traquéia e administração intraamniótica de drogas promoveram reversão destas alterações. Nas artérias com diâmetro externo ≤ 100µm a hérnia diafragmática congênita provocou aumento da espessura da camada média e na contagem de núcleos e decréscimo da densidade de fibras elásticas. A ligadura de traquéia e a administração intraamniótica de surfactante ou dexametasona reverteram estas alterações embora esta última não tenha tido efeito sobre a densidade de fibras elásticas.

**CONCLUSÃO:** A hérnia diafragmática congênita provoca diferentes alterações estruturais nas pequenas e grandes artérias pulmonares. A administração intraamniótica de surfactante ou dexametasona tem efeitos positivos nestas alterações estruturais, comparáveis à ligadura de traquéia.

**DESCRITORES:** **Hérnia diafragmática congênita. Hipertensão pulmonar persistente. Hipoplasia pulmonar. Surfactante. Glicocorticóide.**

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Received for publication on March 19, 2001.