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Original Article

Liver Growth Factor Improves Pulmonary Fibrosis Secondary to Cadmium Administration in Rats

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ABSTRACT

Introduction: Liver growth factor (LGF) is a liver mitogen with regenerating and anti-fibrotic activity even at extrahepatic sites. We used LGF in a lung fibrosis model induced by cadmium chloride ($CdCl_2$), to study its antifibrotic capacity.
Methods: Forty-two male Wistar rats were administered a single dose of 0.5ml/rat of CdCl2 0.025% (n = 21) or the same volume of saline (control group, n = 21). After 35 days, once a lesion was established, we started a 3 week treatment with LGF, after which we determined lung function —inspiratory capacity (IC), lung compliance (LC), forced vital capacity (FVC) and expiratory flow at 75% (FEF75%)—, lung morphometry —alveolar internal area (AIA), mean linear intersection (LM)—, and collagen (both by Sirius red and hydroxyproline residues) and elastin contents.
Results: Pulmonary fibrosis in $CdCl_2$ rats was characterized by a marked decrease in pulmonary function with respect to healthy controls —reductions of 28% in IC, 38% in CL, 31% in FVC, and 54% in FEF75%— which was partially recovered after LGF injection —18% IC, 27% CL, 19% FVC and 35% FEF75%—; increase in collagen and elastin contents —165% and 76%, respectively, in CdCl2 rats, versus 110% and 34% after LGF injection—; and increases in AIA and LM, partially inverted by LGF.
Conclusions: Together, these data seem to demonstrate that LGF is able to improve lung function and partially inverts the increase in lung matrix proteins produced by $CdCl_2$ instillation.

El factor de crecimiento de hígado mejora la fibrosis pulmonar inducida tras la administración de cadmio en ratas

Palabras clave:
Factor de crecimiento de hígado
Fibrosis
Función pulmonar
Morfometría pulmonar
Colágeno

RESUMEN

Introducción: El *liver growth factor* (LGF, 'factor de crecimiento de hígado') es un mitógeno con actividad regeneradora y antifibrótica con actividad incluso en localizaciones extrahepáticas. En este trabajo se administró LGF en un modelo de fibrosis pulmonar inducido con cloruro de cadmio ($CdCl_2$) para estudiar su capacidad antifibrótica.
Métodos: Se instilaron 42 ratas Wistar macho con 0,5 ml/rata de $CdCl_2$ al 0,025% (n = 21) o de salino (n = 21). Transcurridos 35 días y una vez establecida la lesión se realizó el tratamiento con LGF y posteriormente el análisis de los parámetros funcionales —capacidad inspiratoria (CI), complianza pulmonar (CL), capacidad vital forzada (CVF) y flujo espiratorio forzado al 75% (FEF75%)—, morfometría —área interna alveolar y distancia media entre paredes alveolares— y contenido en colágeno y elastina.
Resultados: La fibrosis pulmonar originada mediante $CdCl_2$ se caracterizó por un marcado descenso de la función pulmonar en comparación con los controles. Se redujo un 28% la CI, un 38% la CL, un 31% la CVF y un 54% la FEF75%, descenso que se revirtió parcialmente tras la inyección de LGF —el 18% en CI, el 27% en CL, el 19% en CVF y el 35% en FEF75%—. Además, se observó un incremento en la cantidad de colágeno y elastina del 165 y el 76%, respectivamente, en las ratas del grupo $CdCl_2$ frente a un 110 y un 34% tras la inyección de LGF.
Conclusiones: Estos datos demuestran que el LGF mejora la función pulmonar y revierte parcialmente el incremento de las proteínas de matriz pulmonar producido por la instilación con $CdCl_2$.

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Introduction

Pulmonary fibrosis is a conditioned characterised by a progressive deterioration of the pulmonary function.[1] There are several animal models which attempt to reproduce this condition; perhaps the most extended is bleomycin induction (a fibrogenic agent widely employed for the study of the initial stages of the condition) since it is inverted if the agent is not continuously administered.[2] For the study of the fibrotic stages, the assessment should take place in the second and third weeks from the moment of induction. For this, there are more effective experimental models, such as cadmium chloride ($CdCl_2$) by single dose endotracheal induction in animals which causes pulmonary inflammation with fibrotic activity accompanied by an alveolar space enlargement recalling human centriacinar emphysema,[3] but with a predominance of fibrosis. This agent induces a functional pattern characterised by a decrease in volume, pulmonary distension and expiratory flow[4,5] as well as an increase in proteins on the lung matrix, collagen and elastin.[5,6] Frankel et al[7] substantiated that this model reproduced many of the features of fibrotic response observed in humans and in other models of animal fibrosis. The acute inflammation dropped to close to normal values towards the seventh day, while the amount of collagen remained high and the lung volume[7,8] descended.

The liver growth factor (LGF) is a liver mitogen composed of an albumin-bilirubin complex[9,10] showing activity either *in vivo* or *in vitro*. This factor, purified by our group,[11] was proven in a model of CCI_4 induced cirrhosis; the LGF markedly decreased the accumulation of extra-cellular matrix components (collagen), restored the serum enzymes, the structural integrity and the necrotic tissue, reduced the abscesses and improved haemodynamics.[12] We have recently proven the anti-fibrotic activity of the LGF in bile duct-ligated rats, in which a marked descent in the accumulation of extracellular matrix was produced, recovering its hepatic function as well as reducing key factors in the extension of the fibrosis, such as tumour growth factor (TGF-,).[13] The LGF anti-fibrotic action is measured by the decrease in activation of stellate cells and secreting cells in the extracellular matrix.[14] Subsequent studies have also proven that the LGF is able to reduce both fibrosis in the carotid artery and collagen in the wall of this artery by 50% after 2 weeks of treatment, causing substantial regeneration of the arterial wall, lowering blood pressure and improving vascular function in a model of spontaneously hypertensive rats.[15] Similarly, the LGF was able to stimulate dopamine terminal sprouting, partially restore motor function in rats with Parkinson's disease[16] as well as stimulate the generation of new neurons and mobilisation of neurons.[17]

The LGF mitogenic activity in rat liver is measured by the increase in mRNA expression of the tumour necrosis factor (TNF-·), and the main targets of the LGF are the portal endothelial cells, in which a TNF-· expression increase has been observed. Furthermore, the LGF also stimulates TNF-· secretion in endothelial cell cultures, however, it does not cause an increase in the expression of intercellular adhesion molecule I (ICAM-1) or vascular adhesion molecule I (VCAM-1).[18]

By considering the LGF effect on the fibrosis of other systems, this study proposes a preliminary study on the potential therapeutic effect in a model of lung fibrosis induced with $CdCl_2$. The administration of this factor, once the fibrosis model is established, displays preliminary data of the LGF therapeutic action, obtains improvements in lung function and partially inverts the deposit of matrix proteins.

Methods

1. *Materials and animals:* The Animal Experimentation Committee at the Jiménez Díaz-CAPIO Foundation approved all the experiments performed on animals. Forty-two male Wistar rats (220-230g) were administered a single dose via endotracheal induction of saline (control group, n = 21) or 0.5ml/rat of $CdCl_2$ at 0.025% (n = 21). After instillation, the rats were returned to their cages, where they were administered H_2O and food *ad libitum*. After 35 days, a group of 15 rats was sacrificed (8 control group and 7 $CdCl_2$) to confirm the presence of fibrosis. The remaining animals induced with $CdCl_2$ were divided into 2 new groups and treated with 6 intraperitoneal (i.p.) doses (2 injections/week, for 3 weeks) of saline ($CdCl_2$ group, n = 7) or 5µg/rat of LGF (LGF group, n = 7). Fifty-five days after induction, we compared the $CdCl_2$ and LGF groups on a functional, morphometric and biochemical level. Furthermore, a group of control rats was treated with LGF (control group + LGF) and this group was included in the comparisons.

2. *Lung function tests:* The functional study was performed in a respirator for small animals (Harvard).[5] The rats were anesthetised with sodium pentothal (60mg/kg of weight, i.p.), they were tracheotomised, placed in a plethysmograph and connected to a cannula to allow communication with the respirator. As soon as the rats were connected to the respirator, they were paralysed with 0.2mg of pancuronium bromide and artificially ventilated. Changes in inspiratory capacity (IC) (ml), lung compliance (LC) (ml/cm of H_2O), forced vital capacity (FVC) (ml) and forced expiratory flow at 75% (FEF75%) (ml/s) were then determined.

The IC was considered as a change in the pulmonary volume when an airway pressure of 30cm H_2O was reached. For the quasi static lung pressure-volume curves, the animals were insufflated with air up to an airflow pressure of 30cm H_2O and exit forced to a constant flow of 1ml/s until a residual volume was reached. Compliance was considered as the highest point in the pressure-volume curve during expiration. In the end, flow-volume curves were induced, the rats were insufflated with air to a pressure of 30cm H_2O and forced expiration provoked using a vacuum pump (Emerson) at a pressure of –(40cm H_2O). From these curves, the FVC and the FEF75% of the FVC are determined.

3. *Morphometry:* After the functional study, the animals were sacrificed with an overdose of pentobarbitone and the cardiopulmonary block was removed. The left lung was weighed and immediately frozen to perform the corresponding biochemical determinations. The right lung was set applying a 10% formaldehyde solution at a constant pressure of 25cm H_2O for 24 hours. After setting, 3 lobes were taken, added to paraffin and 5µm thick cuts were made and stained with haematoxylin and eosin for the morphometric study and Sirius red for the collagen deposit quantification. Using an image analysis program for samples stained with haematoxylin and eosin, the alveolar internal area (AIA) (μm^2) was calculated as well as the horizontal and vertical mean linear intersection (LM) (µm). The collagen content was quantified in the samples stained with Sirius red and the birefringence in the collagen-rich areas was compared with the total area of tissue of each microscopic field. The images were visualised with a video camera (Leica DC 100; Leica Microsystems) with a resolution of 782x582 pixels, adapted to a microscope (Olympus BX40). The different fields were quantified using a 10? lens and a 0.5? video camera adaptor. Leica Qwin software was used to analyse the images and the AIA, LM and birefringence calculated. Each datum represents the average value of at least 18 fields selected randomly in different cuts.

4. *Biochemical analysis of collagen and elastin:* Collagen quantification was performed by measuring the hydroxyproline (HYP) residue content. Previously, the left lung was homogenised and chloramine-T was added after acid hydrolysis to induce oxidization. The Erlich (Sigma Aldrich) reagent was then added and once the reaction developed, absorbency at 560nm[19] was assessed

for each of the samples. The elastin was purified through tissue homogenisation with phosphate buffered saline (PBS) and trichloroacetic acid (TCA) at 10%, centrifugation and treatment with TCA at 5% for 30 min at 90 °C. This last precipitation (purified elastin) was treated with porcine pancreatic elastase type III (Sigma Aldrich) for 4 hours at 25 °C. The elastin was measured with a colorimetric method and bicinchoninic acid (BCA Protein Assay Kit; Pierce, Promega) was used. HIP (Sigma Aldrich) and bovine neck ligament elastin (Sigma Aldrich) were used as reference standards. Results were expressed in HIP or elastin milligrams for the left lung.

5. *Liver growth factor isolation and purification:* The LGF was purified of rat serum according to the previously described procedure.[20] The purity and absence of other growth factors or pollutants in the LGF preparation were also performed using standardised criteria.[11] The LGF preparations were freeze-dried and kept at 4 °C until use, when the different aliquots in saline were dissolved for i.p. injection (the LGF is equally active in i.p. and i.v. injection). Before using the LGF in these experiments, its activity was checked *in vivo* at different doses through injection in normal rats to establish the dose at which greater stimulation of DNA synthesis in the liver is produced and for this the incorporation of tritiated thymidine (New England Nuclear; Dreieich, Germany) was determined in the DNA.[11] In accordance with previous studies, it was considered that the optimum dose of LGF was 0.5 µg/rat.[15]

6. *Statistical analysis:* All the data expressed as a mean ± standard error of the mean (SEM). Comparisons were performed with ANOVA. Fisher's least significant difference method was used to analyse the differences between means.

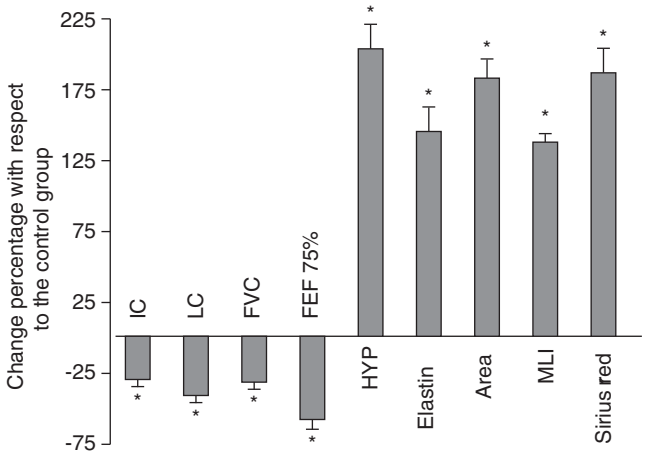

Figure 1. Changes in all the variables analysed in the cadmium chloride group 35 days later, compared to the control group. AIA: alveolar internal area; IC: inspiratory capacity; LC: lung compliance; FVC: forced vital capacity; FEF75%: forced expiratory flow at 75%; HYP: hydroxyproline; LM: mean linear intersection. *p < 0.05 versus control. All data expressed as percentages with respect to the control group.

Results

Thirty five days after inducing the lesion (fig. 1, table 1), a significant descent was detected in all the functional variables studied (IC, LC, FVC and FEF75%) with respect to the values obtained in the control group. On the other hand, a greater increase, also significant, was observed in the protein content of the extracellular matrix, usually found in pulmonary fibrosis, as well as a marked increase in the morphometric variables (AIA and LM) which recall the typical enlargement of the airways of emphysema.

To confirm that the LGF does not present negative effects in the healthy animals, 7 rats in the control group were administered the same treatment as the LGF group. No differences were found in relation to the control group (table 2). Neither were differences found in any of the parameters studied (table 3) between days 35 and 55 within the group of rats to which $CdCl_2$ had been administered, which confirms that the induced lesion was stable and permanent in the period where treatment with LGF was applied.

1. *Pulmonary function*: The changes in IC, LC, FVC and FEF75% in the study groups are shown in figure 2. As previously mentioned, the pulmonary fibrosis induced with $CdCl_2$ produced a significant descent in IC, LC, FVC and FEF75% in comparison with the control rats. The administration of LGF brought all the functional variables to values near normality, with statistically significant variations in IC, FVC and FEF75%, but not in LC (LGF versus $CdCl_2$).

2. *Pulmonary collagen and elastin:* The $CdCl_2$ caused a significant increase in the collagen and elastin contents in the CdCl2 group compared to the healthy rats. After the LGF treatment, the lung content in collagen was less than in the rats that received $CdCl_2$, and this difference was important when assessed in terms of HYP residues (fig. 3). We also detected a notable descent in the pulmonary elastin content in the LGF group in comparison to the rats that received $CdCl_2$ (fig. 3).

Figure 4 shows an increase in the collagen deposit, analysed with birefringence in the collagen-rich areas of the Sirius red staining. The LGF partially inverted the lung matrix deposits, with a tendency to reach normal values, but with no statistical significance.

3. *Morphometry:* The morphometric analysis of the LM and AIA display a significant increase in these variables in the $CdCl_2$ group versus the healthy controls. After treatment with LGF, a slight reduction in the LM and AIA was observed, but with no statistical importance (fig. 5). Figure 6 displays microscope images of a healthy lung and another instilled with $CdCl_2$, where the increase in alveolar size and the peribronchial fibrosis present in the affected group are evident.

Discussion

In this article, we show that the model of $CdCl_2$ induced lung lesion has a fibrotic component characterised by the decrease of the functional variables as well as an increase in the matrix protein deposits and also, an emphysematous component due to the existence of enlarged alveoli observed in the morphometric analysis. Based on this model, established 35 days after the $CdCl_2$

Table 1
Functional, morphometric and biochemical data 35 days after inducing the lesion[a]

	IC, ml	LC, ml/cm H_2O	FVC, ml	FEF75%, ml/s	HYP, mg/lung	Elastin, ml/lung	AIA, μm_2	LM, µm	Sirius red, birefringence/ total tissue, %
C	16.3 ± 0.6	1.3 ± 0.07	16.3 ± 0.6	75.7 ± 6.5	1.5 ± 0.2	13.8 ± 1.4	5,362 ± 842	51 ± 3	1.3 ± 0.2
$CdCl_2$	11.5 ± 0.7*	0.8 ± 0.09*	11.1 ± 0.7*	33.4 ± 6.0*	3 ± 0.6*	19.4 ± 2.6*	9,616 ± 842*	68 ± 3*	2.4 ± 0.2*

AIA: alveolar internal area; $CdCl_2$: cadmium chloride; IC: inspiratory capacity; LC: lung compliance; FVC: forced vital capacity; FEF75%: forced expiratory flow at 75%; H_2O: water; HYP: hydroxyproline; LM: mean linear intersection.

*p < 0.05 versus control.

Table 2
Comparison between the control group and the control group + liver growth factor in every variable

	IC, ml	LC, ml/cm H_2O	FVC, ml	FEF75%, ml/s	HYP, mg/lung	Elastin, ml/lung	AIA, μm_2	LM, µm	Sirius red, birefringence/ total tissue, %
$CdCl_2$. 35 days	11.5 ± 0.7	0.7 ± 0.09	11.1 ± 0.7	33.4 ± 6	3.0 ± 0.2	19.4 ± 2.6	9,616 ± 842	68 ± 3	2.4 ± 0.2
$CdCl_2$. 55 days	11.8 ± 0.5	0.7 ± 0.06	11.5 ± 0.6	35.4 ± 4.2	3.8 ± 0.2	21.6 ± 1.2	1,0931 ± 1,056	69 ± 2	3.1 ± 0.2

No statistical differences are observed between the groups.
AIA: alveolar internal area; $CdCl_2$: cadmium chloride; IC: inspiratory capacity; LC: lung compliance; FVC: forced vital capacity; FEF75%: forced expiratory flow at 75%; H_2O: water; HYP: hydroxyproline; LM: mean linear intersection.

Table 3
Comparison of the functional, morphometric and biochemical data 35 and 55 days after the administration of cadmium chloride

	IC, ml	LC, ml/cm H_2O	FVC, ml	FEF75%, ml/s	HYP, mg/lung	Elastin, ml/lung	AIA, μm_2	LM, µm	Sirius red, birefringence/ total tissue, %
C	16.5 ± 0.5	1.2 ± 0.06	16.7 ± 0.63	77.4 ± 4.6	1.4 ± 0.2	12.3 ± 1.3	3,981 ± 1,141	46 ± 2	1.5 ± 0.2
C+LGF	16.8 ± 0.5	1.31 ± 0.06	16.8 ± 0.5	79.8 ± 6.4	1.6 ± 0.3	13.8 ± 1	4,154 ± 551	47 ± 2	1.9 ± 0.2

No significant differences were found between the groups.
AIA: alveolar internal area; IC: inspiratory capacity; LC: lung compliance; FVC: forced vital capacity; FEF75%: forced expiratory flow at 75%; H_2O: water; HYP: hydroxyproline; LGF: liver growth factor; LM: mean linear intersection.

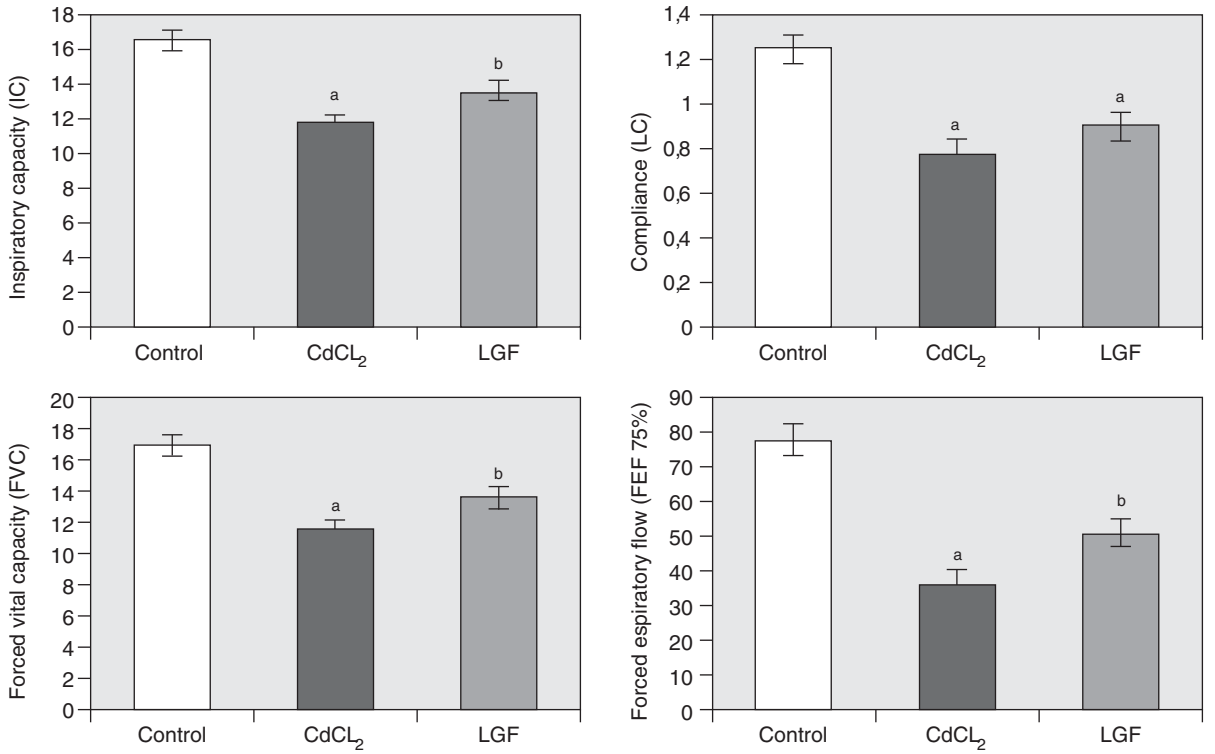

Figure 2. Changes in the functional variables in all the experimental groups. Control group (n = 6), CdCl2 group (n = 7) and LGF group (n = 7). CdCl2: cadmium chloride; IC: inspiratory capacity; LC: lung compliance; FVC: forced vital capacity; FEF75%: forced expiratory flow at 75%; LGF: liver growth factor. [a]p < 0.05 versus control. [b]p < 0.05 versus $CdCl_2$.

instillation, treatment with LGF partially inverts the induced fibrotic lesion. This statement is supported by the improvement in most of the functional variables (IC, FVC and FEF75%) and the notable decrease in the lung matrix proteins (HYP and elastin) after the LGF treatment. The rats instilled with $CdCl_2$ displayed a descent in the functional variables and an increase in the lung matrix protein levels similar at 35 and 55 days after instillation, so we can consider the lesion caused by the $CdCl_2$ as permanent and, therefore, the effects produced by the LGF as therapeutic. To the best of our knowledge, this is the first experimental study in which an antifibrotic treatment partially restores normal lung function of a previously established lesion.

Several authors have described that $CdCl_2$ induced pulmonary fibrosis is characterised by the functional damage, including a descent in the vital capacity, compliance and forced expiratory flow as well as an increase in the lung matrix protein content[4,6,21] and

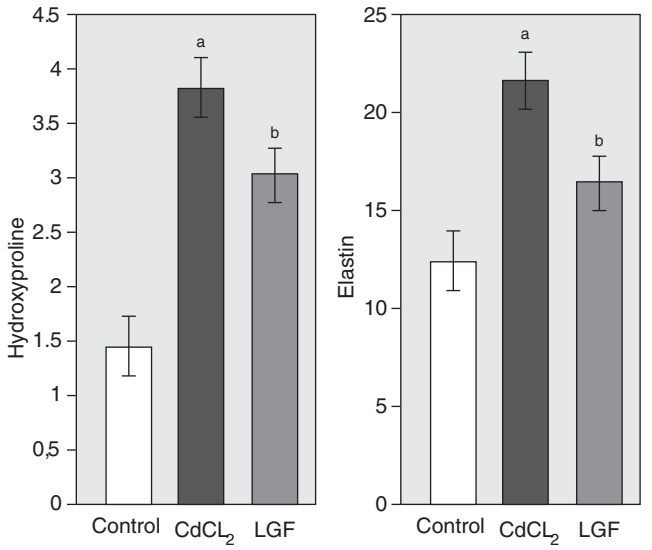

Figure 3. Changes in the biochemical variables in all the experimental groups. Control group (n = 6), $CdCl_2$ group (n = 7) and LGF group (n = 7). Hydroxyproline (mg/lung) and elastin (mg/lung) $CdCl_2$: cadmium chloride; LGF: liver growth factor. [a]p < 0.05 versus control. [b]p < 0.05 versus $CdCl_2$.

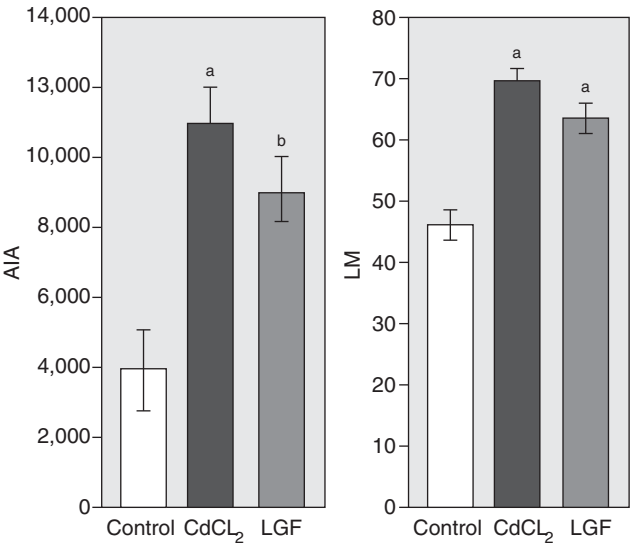

Figure 5. Changes in the morphometric variables (alveolar internal area [mm^2] and mean linear intersection [mm]). Control group (n = 6), $CdCl_2$ group (n = 7) and LGF group (n = 7) $CdCl_2$: cadmium chloride; LGF: liver growth factor. [a]p < 0.05 versus control. [b]p < 0.05 versus $CdCl_2$.

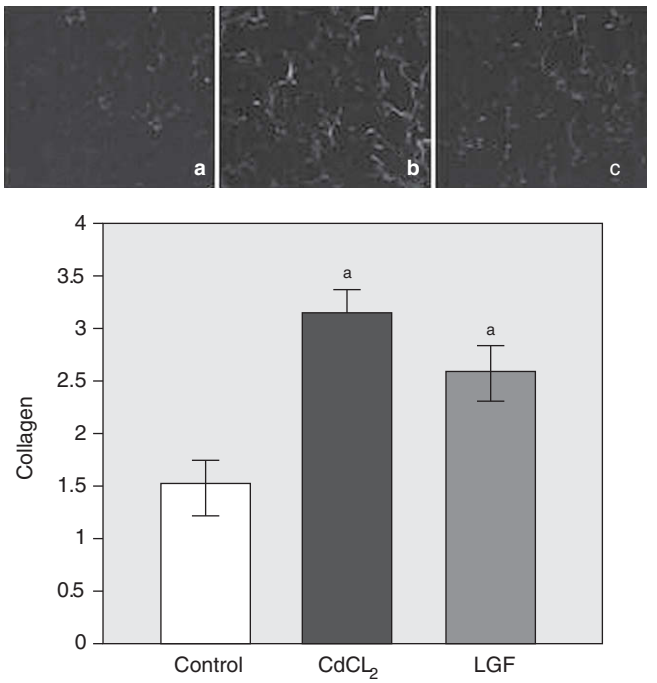

Figure 4. Estimation of collagen content through birefringence with Sirius red. A) Representative case of a normal rat lung. Control group (n = 6). B) Representative case of cadmium chloride ($CdCl_2$) induced pulmonary fibrosis. $CdCl_2$ Group (n = 7). C) Representative case of a lung with $CdCl_2$ induced fibrosis treated with liver growth factor (LGF). A reduction in the fibrotic areas is observed. LGF group (n = 7). Images taken with a 100x augmentation. Data in birefringence % / total tissue. [a]p < 0.05 versus control. [b]p < 0.05 versus $CdCl_2$.

centriacinar emphysema appears.[22] Even though the LGF does not reduce alveolar enlargement caused by the $CdCl_2$ instillation in any significant manner, although there is a tendency of improvement that could open a new line of study on the track towards the effect of this growth factor in the emphysema.

This study has many similarities to the prior one carried out by Heili Frades et al. In both cases, similar lung lesions have been found that affect the function, alveolus size and extracellular matrix protein levels compatible with centriacinar emphysema and peribronchial fibrosis; in this model the treatment with N-acetylcysteine (NAC) partially improved the pulmonary function deterioration, observed in all the functional variables measured (IC, FVC and FEF75%), except in the LC. The same occurs with the collagen deposit measured by the HYP residues, elevated due to the $CdCl_2$ induction and partially restored by the NAC treatment. The response pattern observed with the $CdCl_2$ is very similar to this study, including the responses to treatment. The NAC improves the fibrotic lesion, though the difference lies in the administration method; while this antioxidant agent is administered in a concomitant manner to the induction of the lesion, the LGF is administered once this has been established and stabilised.

The case of NAC is not the only one in medical literature due to the prevalence of this disease and the difficulty of finding effective therapeutic targets to fight it,[23] makes the search for treatment continuous. There are many studies carried out in bleomycin models with antifibrotic agents, such as deferoxamine, the hepatocyte growth factor, the keratinocyte growth factor, interferon and gingko biloba, EM703, IMD-0354 and C-type natriuretic peptide,[1,24-30] and even studies that approach another type of treatment such as cellular treatment based on the instillation of alveolar type II cells as a regeneration vehicle of damaged tissue.[31] Although these agents have the capacity of reducing fibrosis, they also have the limitation that the antifibrotic agent is added concomitantly to the development of the disease, which hinders its therapeutic use since the condition is diagnosed, in the majority of cases, once it has been established.

According to previous publication, the cellular inflammation is not a key factor after the first days of $CdCl_2$ instillation.[7] Since the LGF was administered once the lesion was established, it is unlikely that the LGF activity is due to an anti-inflammatory effect and partially inverts the fibrosis, however, we observe that the LGF has a considerable free radical abduction activity.[32] Our group recently demonstrated that the antifibrotic capacity of LGF is measured by the partial inhibition of the TGF-β, and the transformation of

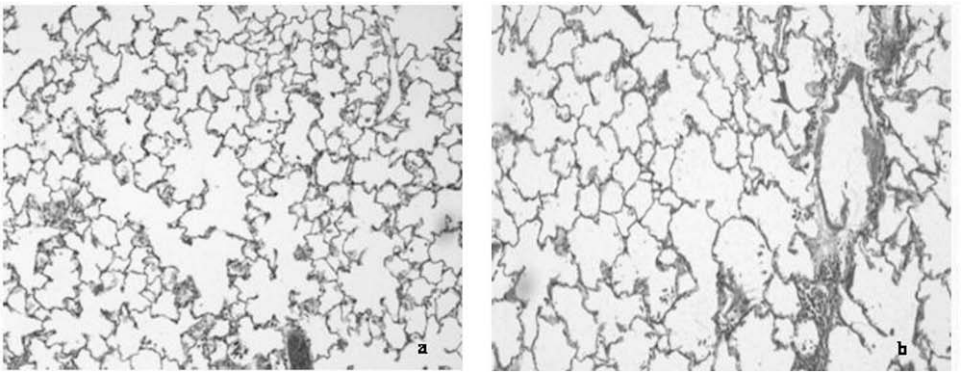

Figure 6. Morphological changes. A) Representative case of a healthy lung. B) Representative case of a cadmium chloride instilled lung showing centriacinar emphysema with peribronchial fibrosis. Stained with haematoxylin and eosin. Images taken with 100x augmentation. $CdCl_2$: cadmium chloride.

myofibroblasts,[13,14] both key in the process of extracellular matrix deposit. However, it is also possible that the LGF chooses mother cells and introduces the proliferation of the endothelial and alveolar cells, as other growth factors do.[27,33-35] Altogether, these observations indicate the need to carry out new studies to determine the action mechanism of the LGF in the repair of lung fibrosis.

To conclude, this study shows that LGF treatment administered on a lung fibrosis previously established through the administration of $CICl_2$, was able to partially invert the pulmonary fibrosis, improve lung function and invert the increase in lung matrix proteins. Pending determination of the action mechanism that facilitates this response, the possibility of inverting a previously established pulmonary fibrosis opens the possibility that LGF can be applied in the future to pulmonary fibrosis treatment.

Conflict of Interest

The authors declare that they have no conflicts of interest.

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