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ORIGINAL ARTICLE

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Retinoic acid rescues lung hypoplasia in nitrofen-induced hypoplastic foetal rat lung explants

Abstract There is increasing evidence to suggest that the retinoid pathway is involved in the pathogenesis of congenital diaphragmatic hernia (CDH). We hypothesised that retinoids are involved in the pathogenesis of associated pulmonary hypoplasia in CDH and therefore designed this study to investigate the effects of retinoid acid on nitrofen-induced hypoplastic lungs. Pregnant rats were exposed to either olive oil or 100 mg nitrofen on day 9.5 of gestation. Foetal lungs were harvested on embryonic day 13.5 and were cultured for 96 h with or without exogenous retinoic acid (RA) (1 μM) added daily to the culture medium. Lungs were divided into four study groups: control ($n=31$); control + RA ($n=19$); nitrofen ($n=19$); and nitrofen + RA ($n=12$). Lung growth was assessed in each group by measuring branching morphogenesis, total DNA content and the proportion of proliferating cells stained by immunohistochemistry. One-way ANOVA test was used for statistical analysis. Retinoic acid significantly increased the growth of nitrofen-induced hypoplastic lungs, whilst growth of control lungs did not change. The number of lung buds and lung area of nitrofen-exposed hypoplastic lungs after 96 h of culture significantly increased after the addition of RA compared to the non-treated hypoplastic lungs (25.75 ± 6.47 vs 15.11 ± 3.29 and 0.98 ± 0.18 mm^2 vs 0.65 ± 0.13 mm^2, respectively; $P < 0.0001$). Lung perimeter was also higher when RA was added to hypoplastic lungs compared to the non-treated ones, although it did not reach significance (12.51 ± 2.53 mm vs 11.19 ± 2.56 mm; $P = 0.17$). Conversely, the addition of RA to control lungs did not affect the number of lung buds, lung area or lung perimeter after 96 h in culture compared to the non-treated ones (31.28 ± 4.66 vs 31.81 ± 6.67; 1.29 ± 0.18^2 vs 1.29 ± 0.23 mm^2 and 18.47 ± 3.47 mm vs 17.89 ± 2.94 mm, respectively; $P =$ NS). Retinoic acid also increased the total DNA content and the proportion of proliferating cells in hypoplastic lungs compared to the non-treated ones (2.59 ± 0.58 μg vs 1.96 ± 0.31 μg and $57.89 \pm 9.46\%$ vs $36.76 \pm 8.15\%$, respectively; $P < 0.001$). The addition of RA did not affect either total DNA content or the proportion of proliferating cells in control lungs compared to the non-treated ones (4.04 ± 0.64 μg vs 3.79 ± 0.85 μg and $58.67 \pm 11.23\%$ vs $56.03 \pm 10.36\%$, respectively; $P =$ NS). This study demonstrates for the first time that RA rescues lung hypoplasia in nitrofen-induced hypoplastic lungs. These results suggest that retinoid pathway may be involved in the pathogenesis of associated pulmonary hypoplasia in CDH.

Keywords Retinoic acid · Retinoids · Lung development · Congenital diaphragmatic hernia · Nitrofen rat model · Lung hypoplasia

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Introduction

Severe pulmonary hypoplasia contributes significantly to the mortality and morbidity in newborn infants with congenital diaphragmatic hernia (CDH) [1]. Although our understanding of the pathogenesis of CDH remains incompletely understood, there is increasing evidence to suggest that the retinoids play a crucial role in its pathogenesis [2].

Retinoids are the family of molecules derived from vitamin A. Vitamin A is obtained from the diet in the form of retinyl esters present in animal meat or β-carotene present in vegetables. After absorption through the gut, retynil esters are transported in chylomicrons to the liver for storage, where they are metabolised into retinol [3–5]. Retinol bound to retinol-binding protein is transferred from the liver via blood to target cells. Retinol-binding protein complex binds to cell surface receptors and is internalised. Within the cytoplasm retinol is bound to cellular retinal-binding proteins where it is converted to retinal by retinol dehydrogenase followed by a further dehydrogenation to retinoic acid (RA) using

four cytosolic retinal dehydrogenase (RALDH). Although all of these enzymes have been shown to catalyse these reactions, RALDH 2 has a predominant role in generating RA. Once RA has been synthesised in the cell, it enters the nucleus and establishes or changes the pattern of gene activity by binding to ligand-activated nuclear transcription factors. There are two classes of these transcription factors: retinoic acid receptors (RAR) and retinoid X receptors (RXR). In humans, rats and mice there are three RARs, α, β and γ and three RXRs, α, β and γ. The RAR–RXR heterodimers are the functional units in transducing the retinoid signal at the gene level [3–5].

The first published evidence linking retinoids with CDH came from the examination of pups born to dams with vitamin A deficient diets. Diaphragmatic hernias were present in 25–40% of the offspring of vitamin A deficient dams, with the majority having right-sided hernias [6]. Studies of retinoid receptor double null-mutant mice, lacking both α and β subtypes of RAR, have demonstrated that some offspring have diaphragmatic hernias similar to those observed in human [7]. A small clinical study showed that infants with CDH had 50% less plasma retinol and retinol-binding protein levels compared to levels in healthy infants [8].

An animal CDH model has been produced by the herbicide Nitrofen (2, 4-dichlorophenyl-*p*-nitropheniyl ether). This has a low toxicity in adult rodents but its administration to pregnant rodents between days 8 and 11 after conception results in a high rate of CDH and associated pulmonary hypoplasia to their embryos that is strikingly similar to the human malformation [9]. A study using genetically engineered mice demonstrated a pronounced suppression of the retinoid response element by nitrofen [10]. Using an in vitro assay it has been demonstrated that nitrofen inhibits RALDH 2, the enzyme catalysing the final step in RA production [11]. Administration of large doses of vitamin A antenatally reduced the incidence of nitrofen-induced CDH in rats from 80 to 50% [12]. Moreover, it has been recently demonstrated that the incidence of CDH can be dramatically reduced to 8% if RA, the downstream product of vitamin A, is given to pregnant rats along with nitrofen [13]. Collectively, these data strongly imply that retinoids are candidates for involvement in the pathogenesis of CDH and that nitrofen may act as a teratogen by interfering with the retinoids pathway. Nitrofen model of CDH showed that hypoplasia of the lung precedes the closure of the diaphragm and led to the concept of a "dual-hit hypothesis" that explains pulmonary hypoplasia by two developmental insults, one occurring before the closure of the diaphragm and the second one occurring after it and as a consequence of the actual compression of the lungs by the herniated abdominal viscera [14, 15].

Embryonic lung buds explants can undergo branching morphogenesis in culture [14, 16, 17]. This in vitro lung culture technique is useful to evaluate the process involved in normal lung development and has proved to have many advantages: the developing lung's environment can be precisely controlled while maintaining the architecture and structure of an intact lung unit. The environment can be manipulated by altering the composition of the medium and substances elaborated by the developing lung tissue can be assayed from the medium [14, 16, 17].

We hypothesised that retinoids are involved not only in the pathogenesis of the diaphragmatic defect, but in the associated pulmonary hypoplasia in CDH and therefore designed this study to investigate the effects of retinoid acid on nitrofen-induced hypoplastic lung buds explants.

Materials and methods

Normal and hypoplastic lung explant model

Adults Sprague-Dawley rats were mated overnight. Twelve hours later the presence of spermatozoids in the vaginal smear was verified and was considered as gestational day 0. Pregnant female rats were then randomly divided into two groups. Animals in the experimental group received intragastrically 100 mg of nitrofen (Wako Chemicals, Osaka, Japan) dissolved in 1 ml of olive oil on day 9.5 of gestation, whereas those in the control group received only vehicle. Gestation was resumed until day 13.5, in which the rats were sedated with isofluorane and then killed by intracardiac injection of sodium pentobarbital. The embryos were recovered by caesarean section and whole lungs were dissected free in Hank's buffered saline (HBSS) under a dissecting microscope (Leica S8 APO). The Department of Health and Children approved all the animal experiments (ref. B100/3530) under the Cruelty to Animals Act, 1876; as amended by European Communities Regulations 2002.

Culture system

Normal and nitrofen-exposed lungs were placed on translucent polycarbonate membrane dish inserts (3 μm pore size, 12 mm diameter Transwell®, Costar, UK). The membranes were then placed on culture dishes (12 well culture dishes, Costar, UK) and serum-free Dulbecco's modified Eagle's culture medium (GIBCO BRL-Life Technologies, UK) was added until the lungs were lying at the air–medium interface. Culture medium was added 100 U/ml penicillin and 100 μg/ml streptomycin (GIBCO BRL-Life Technologies, UK) and was changed every 24 h. Lungs were incubated at 37°C in 5% CO_2 for 96 h. Exogenous RA 1 μM (all-*trans*-Retinoic acid, Sigma-Aldrich) was diluted in ethanol and added daily to the culture medium at a final ethanol concentration of 0.4%. Lungs that did not receive RA received only vehicle. Lungs were divided into four study groups:

control ($n=31$); control + RA ($n=19$); nitrofen ($n=19$); and nitrofen + RA ($n=12$).

Morphometry

Cultured lungs were photographed daily on an inverted microscope and the digitalised images were analysed using a software for image analysis (Image J 1.5 Beta 1, Research Services Branch, National Institute of Mental Health, Bethesda, MD, USA). To assess the growth of the specimens, terminal buds were counted on each lung primordium. Image-analysis software was then used to trace the outline of photomicrographs of each primordium and calculate its area and perimeter.

Quantification of DNA content

At 96 h of culture each lung was detached carefully from the culture membrane under a dissecting microscope, was transferred to a 1.5 microcentrifuge tube and stored at −80°C. Total DNA of each lung was extracted using QIAamp DNA Micro Kit (Qiagen, UK). Total DNA content per lung was measured using a spectrophotometer (Nano Drop ND–1000). Each value is the average of three measurements.

Proportion of proliferating cells

To avoid damage of fragile embryonic lungs in culture, lungs were fixed in situ and processed by hand. At 96 h of culture, culture medium was removed from the culture dish and the membranes were rinsed with HBSS. Adequately 4% paraformaldehyde was added to the well and the membrane insert to cover the lung. Lungs were then fixed for 4 h at 4°C. After that, lungs underwent dehydration using ethanol in a graded series of concentration as follows: 30% ethanol for 10 min, 50% ethanol for 10 min, 70% ethanol for 10 min, 90% ethanol for 10 min, 100% ethanol for 10 min twice. 100% ethanol was removed and replaced with a clearing agent (Histo-Clear II, RA Lamb, UK) for 10 min. This step was repeated twice. After that, membrane inserts were added liquid paraffin (58°C) and placed in an oven set at 58°C for 1 h to allow the paraffin to infiltrate the lungs. After the paraffin block was solid 5 μm sections were cut according to the standard procedure, mounted on glass slides and allowed to dry. The proportion of proliferating cells in the lungs was assessed by immunohistochemistry using Ki-67 antibody (Mouse anti-rat monoclonal MIB5, DakoCytomation) and counterstained with Haematoxilin and Eosin. A proliferation index was calculated by assessing the proportion of positively stained cells over the total number of cells. This was established using a cell counter tool incorporated in software for image analysis (Image J 1.5 Beta 1, Research Services Branch, National Institute of Mental Health, Bethesda, MD, USA).

Statistical analysis

All numerical data are presented as means ± standard deviation. Normality of the groups was checked with Kolmogorov–Smirnov test. Differences between the four groups were tested by one-way analysis of variance (ANOVA). Differences between two groups were tested by unpaired t test. Statistical significance was defined as $P<0.05$. Data were analysed using SPSS 11.0 for Windows programme.

Results

Retinoic acid significantly increased growth of nitrofen-induced hypoplastic lungs. Conversely, addition of RA to control lung explants did not produce any significant change (Fig. 1). Retinoic acid significantly increased lung bud count of nitrofen-exposed hypoplastic lungs after 96 h of culture compared to the non-treated ones (25.75 ± 6.47 vs 15.11 ± 3.29; $P<0.0001$, number of buds). The addition of RA to control lungs did not produce any change in lung bud count compared to the control non-treated lungs (31.28 ± 4.66 vs 31.81 ± 6.67; $P=0.7$, number of buds) (Fig. 2a). Lung area of nitrofen-exposed hypoplastic lungs with added RA was significantly bigger after 96 h in culture compared to the non-treated hypoplastic lungs (0.98 ± 0.18 vs 0.65 ± 0.13; $P<0.0001$; mm_2). The addition of RA to control lungs did not produce any change in lung area compared to the control non-treated lungs (1.29 ± 0.18 vs 1.29 ± 0.23; $P=1$, mm_2) (Fig. 2b). Lung perimeter was slightly higher in RA-exposed hypoplastic lungs compared to the non-treated ones, although it did not reach statistical significance (12.51 ± 2.53 vs 11.19 ± 2.56; $P=0.17$, mm). The addition of RA to control lungs did not produce any change in lung perimeter at 96 h of culture compared to the control non-treated lungs (18.47 ± 3.47 vs 17.89 ± 2.94; $P=0.52$, mm) (Fig. 2c). Nitrofen-exposed hypoplastic lungs with added RA had a significantly higher DNA content compared to the non-treated ones (2.59 ± 0.58 vs 1.96 ± 0.31; $P<0.0005$, μg). DNA content of control lungs did not vary when RA was added compared to the non-treated control lungs (4.04 ± 0.64 vs 3.79 ± 0.85; $P=0.28$, μg) (Fig. 3). The proportion of proliferating cells in nitrofen-exposed hypoplastic lungs exposed to RA was significantly higher than in the nitrofen-exposed hypoplastic lungs without added RA (57.89 ± 9.46 vs 36.76 ± 8.15; $P<0.001$, % of total number of cells) and reached the value of the control lungs. Addition of RA to control lungs did not produce any change in the proportion of proliferating cells at 96 h of culture compared to the control non-treated lungs (58.67 ± 11.23 vs 56.03 ± 10.36; $P=0.4$, % of total number of cells) (Figs. 4, 5).

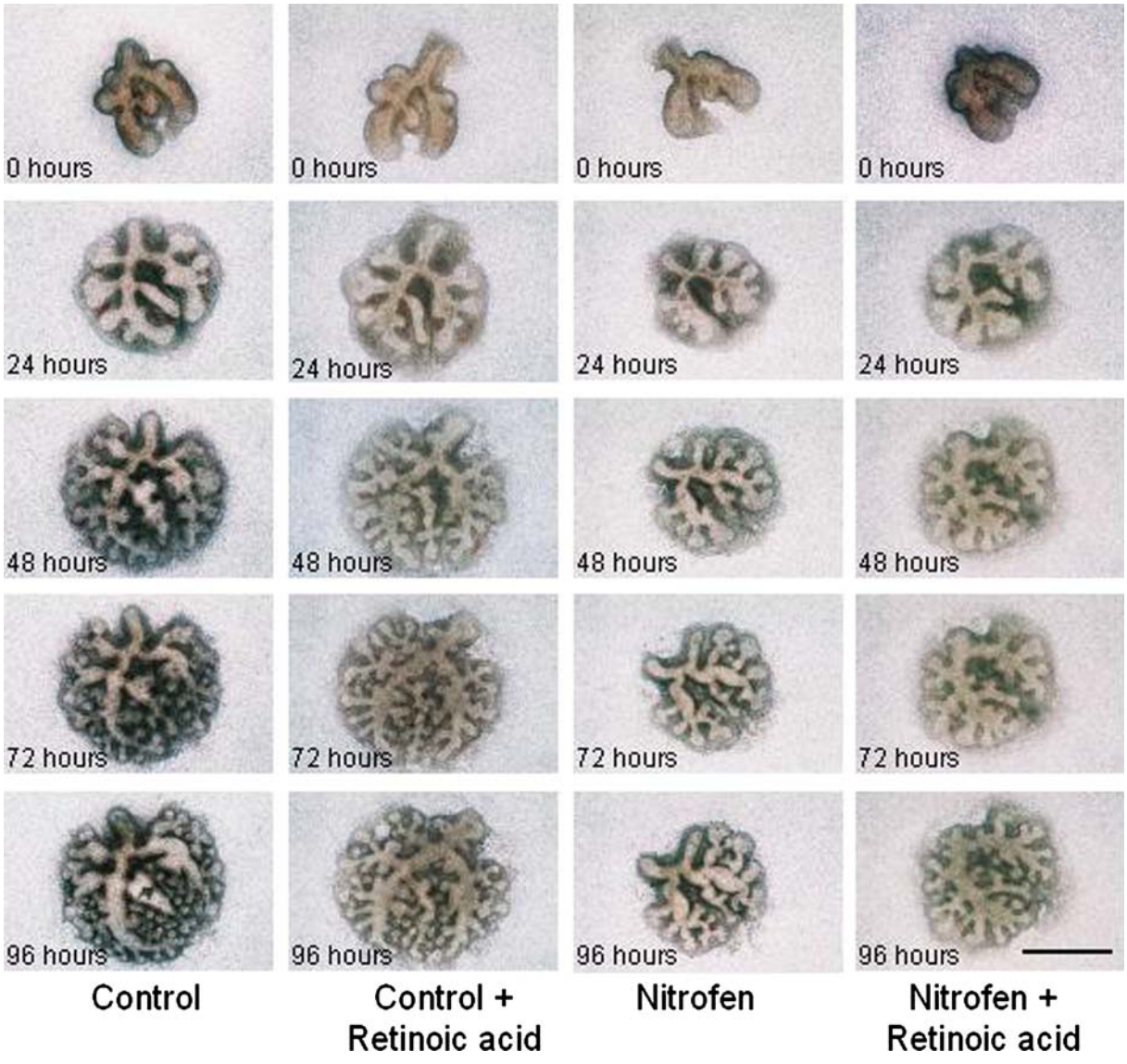

Fig. 1 Representative photographs of embryonic lung explants in culture. Lungs were microdissected from rat embryos at day 13.5 of gestation (0 h in culture) and were cultured for 96 h. Note that the addition of RA increased growth of nitrofen-induced hypoplastic lungs, whereas growth of control lungs remained unchanged. All specimens are at the same magnification: *scale bar* 1 mm

Discussion

Despite remarkable progress in resuscitation and intensive care, the morbidity and mortality rates in CDH remain high [1]. Most newborns with CDH die primarily of severe respiratory failure secondary to pulmonary hypoplasia and pulmonary hypertension. The need to fully comprehend hypoplastic lung development in CDH remains of utmost importance to the search for effective treatments. Traditionally, it was believed that failure of closure of pleuroperitoneal canals resulted in a diaphragmatic defect allowing intraabdominal organs to enter the thoracic cavity, compressing the lungs and resulting in pulmonary hypoplasia [18]. This classical hypothesis has lately become more and more disputed. Much of our current understanding of pathophysiology of CDH originates from experimental studies. The nitrofen-induced rodent model of CDH has been studied for many years and has the advantage that the defect is induced at the stage when the foregut has just separated into the oesophagus and the trachea, providing information about the closure process of the diaphragm and the early stages of lung development [9]. Studies in this model have shown that pulmonary hypoplasia is already present for several days before the closure of the diaphragm takes place during embryonic development [14, 15]. These studies led to the concept of a "dual-hit hypothesis" that explains pulmonary hypoplasia by two developmental insults, one occurring before the closure of the diaphragm and the second one occurring after it

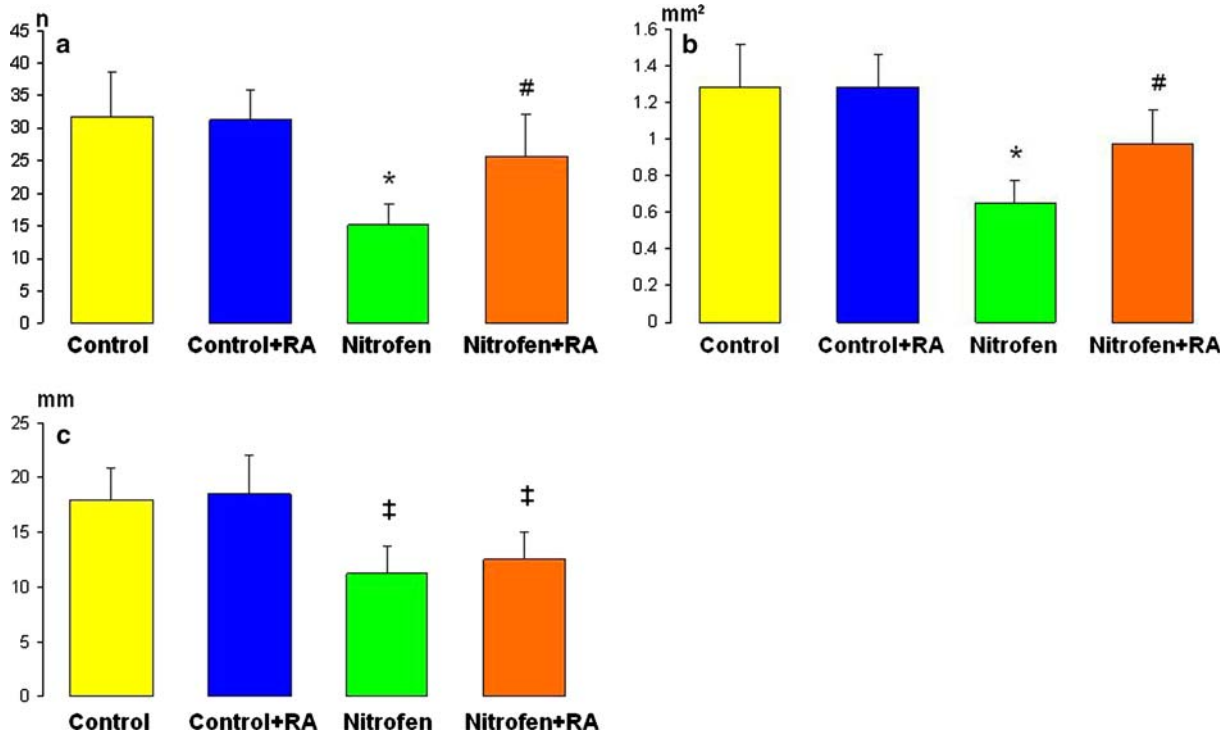

Fig. 2 Morphometric parameters analysed in control lungs, control + RA lungs, nitrofen lungs and nitrofen + RA lungs after 96 h of culture. Lung buds count (**a**), lung area (**b**) and lung perimeter (**c**) (mean ± SD), $*P < 0.001$ versus nitrofen + RA, control and control + RA. $\#P < 0.05$ versus control and control + RA. $\ddagger P < 0.01$ versus control and control + RA

and as a consequence of the actual compression of the lungs by the herniated abdominal viscera [14, 15]. Moreover, Babiuk and Greer [19] have recently shown using *FGF 10* null mutant mice (bilateral lung agenesis) that a diaphragmatic defect could be induced with nitrofen, demonstrating that diaphragm formation is independent from lung formation. According to these recent studies, diaphragmatic defect and lung hypoplasia in CDH may be parallel phenomena.

Numerous studies reveal the role of a retinoid signalling pathway disruption in the pathogenesis of CDH [6, 7, 10–13]. However, most of them have been focused on the diaphragm and the reduction in the incidence of CDH after administration of vitamin A and its derivatives. Recently, Baptista et al. [20] have demonstrated that antenatal vitamin A administration attenuates lung hypoplasia by interfering with early determinants of lung underdevelopment, suggesting that retinoids could also be implicated in the pathogenesis of lung hypoplasia. Isolated embryonic lung buds culture is an excellent tool for studying branching morphogenesis and its modifying factors independently of the formation of other organs. Using this technique we demonstrated for the first time that RA rescues lung hypoplasia in nitrofen-induced hypoplastic lungs. These results strongly suggest that retinoid pathway may be involved not only in the pathogenesis of the diaphragmatic defect in CDH but also in the pathogenesis of associated pulmonary hypoplasia. The increase in lung branching morphogenesis in nitrofen-induced hypoplastic lungs by the administration of RA is consistent with the hypothesis that the decreased RALDH 2 activity can be partially countered by the increase of substrate [13]. The administration of RA bypasses this enzymatic perturbation.

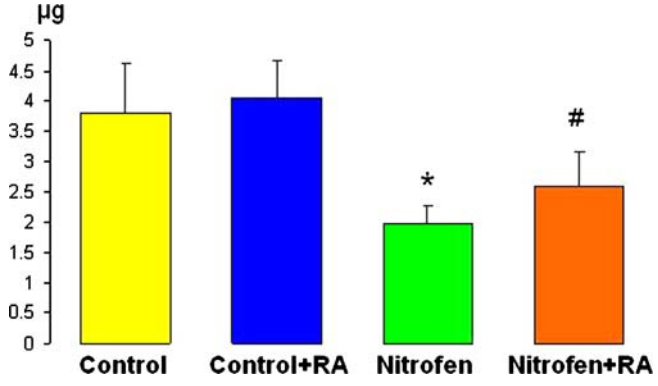

Fig. 3 Total lung DNA content after 96 h of culture in control lungs, control + RA lungs, nitrofen lungs and nitrofen + RA lungs (mean ± SD). $*P < 0.001$ versus nitrofen + RA, control and control + RA. $\#P < 0.05$ versus control and control + RA

Human lung development is a long and complicated process that takes up 90% of the period of gestation and then continues well into childhood and can be divided into five phases: embryonic, pseudoglandular, canalicular, saccular and alveolar. Retinoids play an important role in each of the lung developmental stages [21]. The agenesis of the lung buds seen in embryos under

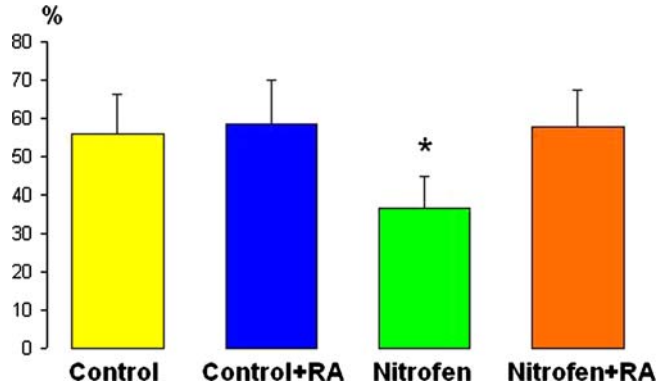

Fig. 4 The proportion of proliferating cells in control lungs, control + RA lungs, nitrofen lungs and nitrofen + RA lungs after 96 h in culture (mean ± SD). $*P < 0.001$ versus nitrofen + RA, control and control + RA

conditions of acute maternal retinol deficiency highlights the importance of retinoids in the stimulation of the initial budding of the lungs [21]. Retinoic acid biosynthesis and the expression of RALDH 2 are detected throughout the layers where the trachea and primitive lungs are forming. Binding proteins (CRBPs) and retinoid receptors (RAR β, RXR α and β) are expressed in the early phases of lung formation [22, 23]. It has recently been demonstrated that a major role for RA in early lung morphogenesis is to selectively maintain mesodermal proliferation and induce FGF-10 expression in the foregut region where the lung forms [24]. Together these data demonstrate the importance of retinoids in the initial budding of the lungs from the foregut. The subsequent pseudoglandular stage of lung development is highly dependent on epithelial–mesenchymal interactions and vitamin A plays a crucial role. Using the determination of RA activity by RARE *lacZ* trasgene expression assays and cultured mouse embryos treated with agonists and antagonist ligands for retinoids receptors, it was reported that RA worked as an inhibitor in distal branching [25]. However, a converse conclusion was reached by Schuger et al. [26], who concluded that there was an increased branching activity of E12 mouse lung explants culture for 48 h. In our study the addition of RA to control lung explants did not appear to affect normal lung branching morphogenesis. During the transition from the pseudoglandular to the canalicular stage of lung development RAR β, RXR α and β are associated with epithelial cell differentiation and structural changes [27]. In the subsequent saccular period, RA stimulates proliferation of the stem cells of the alveolar epithelium through an epithelial growth-factor-mediated pathway [26, 28]. RAR β upregulation is associated with the terminal saccular stage during type I and II epithelial cell differentiations, suggesting its role in the induction of alveolar development [27].

Even though RA significantly increased lung growth in nitrofen-induced hypoplastic lungs in our study, lungs neither reached the size nor had the number of cells (DNA content) of control lungs. It is not clear why RA-exposed lungs do not grow as big as control lungs. It may be that a number of other molecules are also

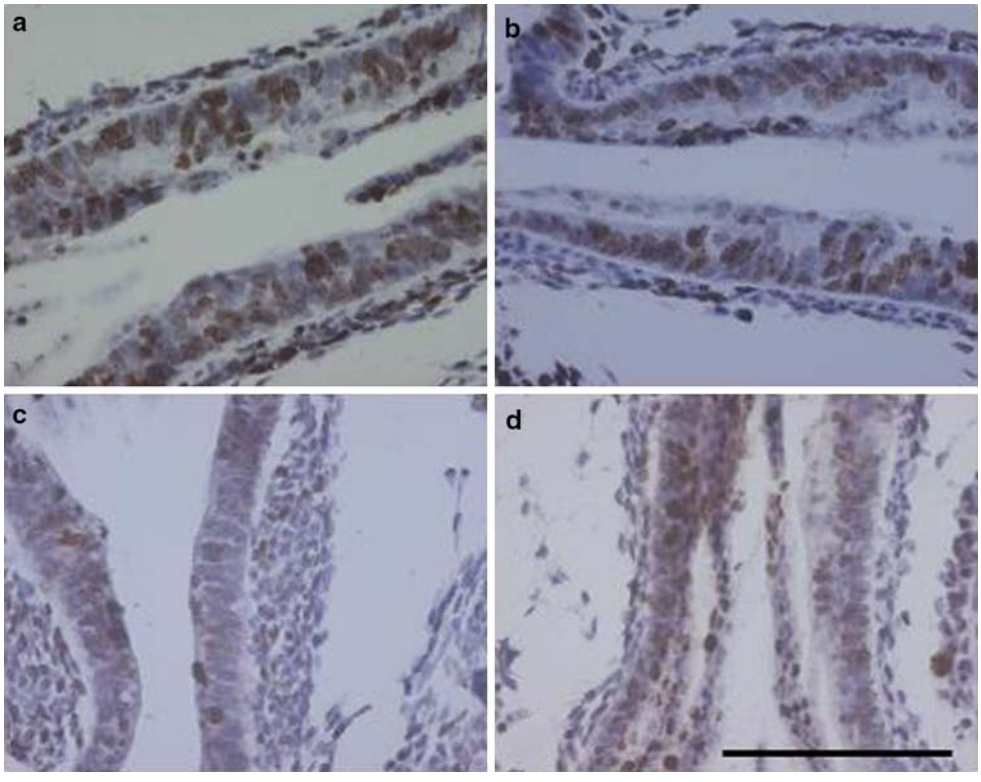

Fig. 5 Representative sections of lungs in control (**a**), control + RA (**b**), nitrofen (**c**) and nitrofen + RA (**d**). Proliferating cells are stained with immunohistochemistry for Ki-67 antigen (*brown*). Note that there are few positively stained cells in the nitrofen group. *Scale bar* 100 μm

involved in the pathogenesis of lung hypoplasia in CDH. The gene expression of members from the steroid/thyroid hormone receptor superfamily have been shown to be downregulated in the nitrofen model of CDH [29, 30]. Some other studies indicate that RXRs and RARs can both serve as robust heterodimers partners with thyroid hormone receptors to regulate thyroid hormone-modulated gene expression [31, 32]. Therefore, the downregulation of the thyroid hormone signalling pathway through altered expression of thyroid hormone receptors may be considered as a potential contributory factor in the pathogenesis of pulmonary hypoplasia in nitrofen-induced CDH [30]. However, the role of thyroid hormones during murine antenatal lung growth appears to be limited, due to the fact that thyroid hormone receptor null mutants do not have a phenotype of severely disturbed antenatal lung development [33]. Therefore, retinoids appear to be the major factor for antenatal modulation of lung growth in the nitrofen model of CDH.

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