Diplomarbeit

Effects of intraamniotically applied IGF-1 and -2 on protein expression in hypoplastic lungs of fetal rats with nitrofen-induced diaphragmatic hernia

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Graz, Juni 2014 *Denise Sarah Plahsnig*

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Zusammenfassung

Zielsetzung: Statistisch gesehen kommt eines von 2500 Neugeborenen weltweit mit einer angeborenen Zwerchfellhernie (CDH) zur Welt. Die Folgen sind eine pulmonale Hypoplasie und Hypertonie, die je nach Schweregrad mit einer hohen Morbidität und Mortalität vergesellschaftet sind. Die Insulin-like growth factors (IGFs) sind Hormone, die durch ihren stimulierenden und regulierenden Einfluss auf das Wachstum und die Differenzierung vieler Gewebearten auch eine wichtige Rolle in der fetalen Lungenentwicklung spielen. In dieser Studie sollte die Wirkung von intraamniotisch appliziertem, humanem rekombinantem IGF-1 (rhIGF-1) beziehungsweise IGF-2 (rhIGF-2) auf die hypoplastischen Lungen von Rattenfeten am Nitrofen-Modell untersucht werden.

Methoden: Trächtigen *Sprague-Dawley-Ratten* wurden am Schwangerschaftstag 9,5 (E9,5) 100 mg in Olivenöl gelöstes Nitrofen verabreicht, die Ratten der Kontrollgruppe blieben unbehandelt. In das Fruchtwasser der jeweiligen Feten der Nitrofen-exponierten Mutterratten wurden an den Tagen 19 und 20 jeweils 0,2 µg rhIGF-1 (IGF-1-Gruppe), rhIGF-2 (IGF-2 Gruppe) oder eine Kochsalzlösung (Placebogruppe) injiziert. Am Tag 21,5 wurden die überlebenden Feten operativ entbunden und auf das Vorliegen einer angeborenen Zwerchfellhernie untersucht. Die linken Lungen von jeweils 10 CDH-positiven Feten der beiden IGF Gruppen, 5 Feten der zugehörigen Placebogruppen und 5 Feten der Kontrollgruppe wurden mittels Immunhistochemie auf die Expression spezifischer Marker der Lungenentwicklung untersucht.

Ergebnisse: Die Proteinexpression von Ttf-1, SMA, Fgf-10, Ki67 und Pdpn war weder in der IGF-1, noch in der IGF-2-Gruppe im Vergleich zur jeweiligen Placebogruppe signifikant verändert. Der Unterschied zwischen den Behandlungsgruppen und der unbehandelten Kontrollgruppe dürfte weitgehend auf die Nitrofenwirkung und die chirurgische Intervention zurückzuführen sein.

Schlussfolgerung: Die intraamniotische Applikation von IGF-1 und -2 hat keine signifikanten Effekte auf die untersuchte Proteinexpression spezifischer Marker der Lungenentwicklung und -proliferation bewirkt.

Abstract

Purpose: One in about every 2500 newborn babies suffers from the consequences of a Congenital Diaphragmatic Hernia (CDH), such as pulmonary hypoplasia and pulmonary hypertension. Insulin-like growth factors (IGFs) are hormones with the ability to stimulate and regulate growth and differentiation of miscellaneous tissues, among, the tissue of fetal lungs. This experimental study aimed to investigate the effects of intraamniotically administered human recombinant IGF-1 (rhIGF-1), respectively IGF-2 (rhIGF-2) on the lungs of rat fetuses with nitrofen induced CDH and resulting lung hypoplasia.

Methods: Timed pregnant *Sprague-Dawley rats* were exposed either to 100 mg nitrofen dissolved in olive oil on E9.5 (treatment groups), or remained untreated (control group). On E19 and E20 0.2 µg of rhIGF-1 (IGF-1 group; n=10), rhIGF-2 (IGF-2 group; n=10) or saline (placebo group; n=5 for each treatment group) were injected into the amniotic fluid of the nitrofen-exposed fetuses. After caesarean delivery on E21.5 surviving fetuses were examined concerning the existence of a CDH, then euthanized and necropsy was performed. The CDH positive left lungs of the 10 fetuses in each of the two IGF-groups, the 5 fetuses in the associated placebo groups and the 5 fetuses in the control group were analyzed by means of immunohistochemistry to evaluate the expression of specific markers for lung development.

Results: Protein expression of Ttf-1, SMA, Fgf-10, Ki67 and Pdpn was neither in the IGF-1 nor in the IGF-2 group significantly changed compared to placebos. The difference between the treatment groups and the untreated controls might largely be due to the effect of nitrofen and the surgical intervention.

Conclusion: Summarizing the results of our study, intraamniotically applied IGF-1 and -2 showed no beneficial effect on the protein expression of specific markers of lung proliferation and differentiation.

Table of contents

DANKSAGUNG **3**

ZUSAMMENFASSUNG **5**

ABSTRACT **6**

TABLE OF CONTENTS **7**

GLOSSARY **9**

LIST OF FIGURES **11**

1 INTRODUCTION **13**

1.1. THE CONGENITAL DIAPHRAGMATIC HERNIA 13

1.1.1. Diagnosis *14*

1.1.2. Prognosis *14*

1.1.3. Prenatal treatment *14*

1.1.4. Postnatal therapy *15*

1.1.5. Ventilation *15*

1.1.6. Treatment of PPHN *16*

1.1.7. Extracorporeal Membrane Oxygenation *16*

1.1.8. Survival and outcome *16*

1.2. THE DEVELOPMENT OF THE DIAPHRAGM IN HUMANS AND RATS 17

1.3. THE DEVELOPMENT OF THE LUNGS 18

1.3.1. The embryonic period (0-5 weeks) *18*

1.3.2. The pseudoglandular period (5-16 weeks) *18*

1.3.3. The canalicular period (16-26 weeks) *18*

1.4. THE MATURATION OF THE LUNGS 19

1.4.1. The saccular period (26 weeks-birth) *19*

1.4.2. Alveolar differentiation (8 months-early childhood) *19*

1.4.3. Pathogenesis of lung hypoplasia *20*

1.5. CDH NITROFEN-MODEL OF THE RAT 20

1.6. THE ROLE OF INSULIN-LIKE GROWTH FACTORS (IGFS) 22

1.7. Reference to this trial *26*

1.8. Antibodies used in immunohistochemistry *27*

1.9. The aim of the study *27*

2. MATERIALS AND METHODS 28

2.1. THE PRACTICAL BIOESSAY 28

2.1.1. The administration of nitrofen 28

2.1.2. Surgical interventions and rhIGF-injections 29

2.1.3. Humane closure (criteria that indicated euthanasia or the abruption of this experiment) 30

2.2. PRACTICAL IMMUNOHISTOCHEMISTRY 30

2.3. ANALYSIS OF TISSUE SECTIONS 32

2.4. STATISTICAL ANALYSIS 33

3. RESULTS OF THE IMMUNOHISTOCHEMICAL STAINING 34

3.1. TTF-1-STAINING 35

3.2. PDPN/T1A-STAINING 37

3.3. SMA-STAINING 39

3.4. FGF10-STAINING 41

3.5. KI67-STAINING 43

4. DISCUSSION 45

4.1. CONCLUSION 49

5. LIST OF REFERENCES 50

Glossary

α-SMA: alpha-smooth muscle actin

ACTH: adrenocorticotropic hormone

ARDS: acute respiratory distress syndrome

CDH: congenital diaphragmatic hernia

ECMO: extracorporal membrane oxygenation

FETO: fetal endoscopic tracheal occlusion

FGF: fibroblast growth factor

Fgf10: fibroblast growth factor-10

HFOV: high frequency oscillation ventilation

IGF: insulin like growth factor

IGFBP: insulin like growth factor binding protein

IGFR: insulin like growth factor receptor

IGF-1: insulin like growth factor 1

IGF-2: insulin like growth factor 2

IGF1-R: insulin like growth factor 1 receptor

IGF2-R: insulin like growth factor 2 receptor

iNO: nitric oxide

IR: insulin receptor

IUGR: intrauterine growth restriction

Ki67: MKI67.

MRI: magnetic resonance imaging

Nsp: number of strong positive cells

NTotal: total number of cells

PCNA: proliferating cell nuclear antigen

Pdpn: Podoplanin

PGE: prostaglandin

PIP: peak inspiratory pressure

PPF: pleuroperitoneal fold

PPHN: persistent pulmonary hypertension of the newborn

proSp: prosurfactant protein

rhIGF-1: recombinant human insulin like growth factor 1

rhIGF-2: recombinant human insulin like growth factor 2

RT-PCR: real time polymerase chain reaction

SD: standard deviation

SP-B: surfactant protein B

TBG: thyroid binding globuline

Ttf-1: thyroid transcription factor-1

T3: triiodothyronine

List of figures

Fig. 1 Embryogenesis of diaphragm defects in CDH © Clugston et al.

Fig. 2 Chemical structure of nitrofen © Roche Lexikon Medizin, 4th edition

Fig. 3 Chemical structure of Triiodothyronine (T3) © Roche Lexikon Medizin, 4th edition

Fig. 4a Ttf-1 expression: Results of the student´s t-test between IGF-1 and the associated placebo group.

Fig. 4b Ttf-1 expression: Results of the student´s t-test between IGF-2 and the associated placebo group.

Fig. 4 (c-d) Microscopic images of the **Ttf-1** immunohistochemical staining.

Fig. 4c Placebo group

Fig. 4d IGF-2 group

Fig. 5a: Pdpn expression: Results of the student´s t-test between IGF-1 and the associated placebo group.

Fig. 5b: Pdpn expression: Results of the student´s t-test between IGF-2 and the associated placebo group.

Fig. 5 (c-d) Microscopic images of the **Pdpn** immunohistochemical staining.

Fig. 5c Placebo group

Fig. 5d IGF-1 group

Fig. 6a SMA expression: Results of the student´s t-test between IGF-1 and the associated placebo group.

Fig. 6b SMA expression: Results of the student´s t-test between IGF-2 and the associated placebo group.

Fig. 6 (c-d) Microscopic images of the **SMA** immunohistochemical staining.

Fig. 6c Placebo group

Fig. 6d IGF-2 group

Fig. 7a Fgf10 expression: Results of the student´s t-test between IGF-1 and the associated placebo group.

Fig. 7b Fgf10 expression: Results of the student´s t-test between IGF-2 and the associated placebo group.

Fig. 7 (c-d) Microscopic images of the **Fgf-10** immunohistochemical staining.

Fig. 7c Placebo group

Fig. 7d IGF-2 group

Fig. 8a Ki67 expression: Results of the student´s t-test between IGF-1 and the associated placebo group.

Fig. 8b Ki67 expression: Results of the student´s t-test between IGF-2 and the associated placebo group.

Fig. 8 (c-d) Microscopic images of the **Ki67** immunohistochemical staining.

Fig. 8c Placebo group

Fig. 8d IGF-1 group

1 Introduction

1.1. The Congenital Diaphragmatic Hernia

One in about every 2500 newborn babies suffers from the consequences of a Congenital Diaphragmatic Hernia (CDH). The malformation of the fetal diaphragm arises in the early development of the embryo, but yet, little is known about the etiology of this defect. The most common theory centers on a failed obturation of the pericardioperitoneal canal by the pleuroperitoneal membrane around the seventh gestational week. If this closure does not occur, the peritoneum and the parietal pleura keep communicating, which leads to CDH, in the majority of cases appearing as the classic postero-lateral “Bochdalek-hernia”. In 85-90% of the cases, the hernia is located on the left side, allowing the stomach, the spleen and parts of the liver to enter the thoracic cavity. This forces the heart in a more ventral position and compresses the lungs by leaving them little space to develop properly. This leads to an insufficient maturation of the lungs, resulting in pulmonary hypoplasia, mainly of the ipsilateral but also of the contralateral lung [1].

Controversial literature claims, that the diaphragmatic hernia might even derive from a primary defect in the lung, but the chicken-or-egg question still has not been fully clarified to the present [2]. Nevertheless, the resulting condition of pulmonary hypoplasia is complex, not only altering the interstitium, but also the airways and the vessels. While the number of alveoli and vessels decrease, their walls thicken, reducing the surface area of gas exchange. A higher amount of interstitial tissue diminishes air space and reduces compliance. At birth, these structural changes often resume in respiratory failure due to hypoxia, hypercapnia and pulmonary arterial hypertension, inducing a right-to-left-shunt, which is worsened by a greater tendency of vasoconstriction [3]. Depending on the size and location of the hernia, severe and less severe cases of CDH can be observed. The mortality can reach 75% in severe cases [1]. CDH often occurs in association with other malformations, including the central nervous system, the heart, the gastrointestinal tract or chromosomal abnormalities.

1.1.1. Diagnosis

Modern prenatal ultrasound enables an early diagnosis, usually between the gestational weeks 16 to 24. Typically the stomach is found in the left chest, shifting the mediastinum to the right. Rare defects located on the right side can be more difficult to diagnose [4]. If a CDH is suspected, the parents should be referred to a specialized center, for an advanced structural ultrasound and possibly a prenatal Magnetic Resonance Imaging (MRI). Thereby associated anomalies can be ruled out or detected, giving the physicians a general comprehension of the condition of the fetus. The parents are counseled and can then base their decision on this early prognostic assessment [3]. The prenatal finding of a CDH does not have to result in a change of gestational guidance or in a planned caesarean section, which, however, may be of advantage in terms of optimization of postnatal treatment including extracorporeal membrane oxygenation (ECMO).

1.1.2. Prognosis

Efforts have been made to identify prognostic factors to base clinical decisions and informed consent on. However, it is a challenge to evaluate the prenatal morphological conditions of the fetal lungs in a way that enables a realistic prediction of the situation after birth. Lung size, resistance in pulmonary vessels as well as type and grade of organ herniation, all have been assessed by means of MRI, 2D or 3D ultrasound and Doppler measurements. To date, the most accepted prognostic indicator is the lung area to head circumference ratio (LHR). The lung area usually expands up to four times more than the head circumference, especially between 12 and 32 weeks of gestation. Measurements taken by ultrasound can be used to define the LHR. The observed LHR is then set in proportion to the expected LHR of a fetus at the same gestational age with normal lungs (O/E LHR). If the O/E LHR is lower than 25%, the chances of survival fall below 15%.

1.1.3. Prenatal treatment

Ambitions to support the lungs of fetuses with CDH in their development in utero have led to attempts of prenatal therapy. The idea of artificially improving prenatal lung growth, has given rise to a treatment called tracheal occlusion. Normally, during pregnancy, the fetal lungs egress fluid causing a positive pressure under the glottis

and thereby inducing lung growth. Pressure can be released by fetal breathing movements. This creates a cycle of growth and cell differentiation, helping the lung to develop and prepare itself properly for its function outside the womb. In case of CDH, a balloon inserted into the trachea can simulate this important step even if natural circumstances can never be imitated. Via fetal endoscopic tracheal occlusion (FETO) a balloon is temporarily placed into the trachea approximately between weeks 26-34 of gestation, preventing the egress of lung fluid and consequently enhancing lung growth by the help of pressure. This technique may improve survival in cases with severe pulmonary hypoplasia, and may ameliorate the outcome depending on the pre-existing lung size and the gestational age. Though only minor maternal complications are reported, the risk for iatrogenic preterm rupture of the membrane must be considered. The long-term morbidity of the survivors does not seem to be affected significantly [3].

1.1.4. Postnatal therapy

While great advances in neonatal care have meliorated the overall survival rate of children born with CDH, the morbidity and mortality still remain high in severe cases of CDH. Early attempts of treatment involved instant surgical repair. Whereas the anatomical defect could be fixed, the insufficient development of the lung could not and no improvement of gas exchange could be reported. Nowadays the focus lies on the improvement of the pulmonary hypoplasia and the vascular abnormalities by means of highly specialized techniques of “gentle” ventilation and surgical intervention is usually deferred until the infant is stable enough.

1.1.5. Ventilation

In the past, newborns with CDH have been ventilated with positive pressure in order to oxygenate them properly. Still, the mortality remained high, because the ventilation injured the hypoplastic lungs even more. Specialized centers all over the world have tried to come up with different approaches for the ventilation of hypoplastic lungs and for the management of the persistent pulmonary hypertension of the newborn (PPHN), since. This includes the use of hyperventilation to provoke alkalosis, in hopes of preventing ductal shunting. It proved to have a negative, even harmful outcome and therefore was discarded. A different strategy of “permissive hypercapnia” was introduced, tolerating a mild hypercapnia, while focusing to keep

the preductal oxygen saturation greater than 85% and the peak inspiratory pressure (PIP) at 25cm H_2O or less. This approach showed a good overall outcome. Nowadays, a technique named High Frequency Oscillation Ventilation (HFOV) has proven itself in practice and ameliorated the survival rate, especially as an interventional approach to prevent lung injuries when the PIP transcends 25cm H_2O on conventional ventilators.

1.1.6. Treatment of PPHN

The best way to diagnose PPHN is to use echocardiography. Thereby, the degree of pulmonary artery pressure can be detected by flattening of the interventricular septum, the existence of a tricuspid regurgitation, and a right-to-left or bidirectional shunting at ductal level. In severe cases, it can be desirable to keep the ductus open to prevent right ventricular failure, when the pressure rises to suprasystemic levels. This can be achieved by prostaglandin (PGE). As long as the preductal saturation is not lower than 85%, the infant's brain is not likely to be harmed. Significant ductal shunting or elevated right ventricular pressures can be treated by inhalation of nitric oxide (iNO), but therapy has to be monitored by echocardiography. Low systemic pressures and possibly right heart failure can be countered with intravenous fluids and inotropic assistance.

1.1.7. Extracorporeal Membrane Oxygenation

In very severe cases of CDH and pulmonary hypertension, ECMO is used to stabilize the infants prior to surgery. Some centers also use it to cope with severe hypoxemia after surgery. While a few institutions have reported improved survival rates, others experienced equally good outcomes without the use of ECMO. On the contrary, morbidity rates have risen since introducing ECMO, including chronic lung diseases and neurological problems, not to mention that some infants don't manage to separate from ECMO. Therefore the indication for an ECMO therapy has to be placed precisely and is not an option for highly cardiopulmonary challenged infants [4].

1.1.8. Survival and outcome

In the last years the survival of infants born with CDH has increased in specified clinics all over the world and reached a plateau at about 70-75%. However, since

early diagnostic possibilities have improved, more pregnancies with CDH fetuses are terminated nowadays. Depending on whether CDH is isolated or associated with other anomalies, the termination rates are between 9 and 51% in the UK for example. This leads to the assumption, that the higher survival rate might be linked to the rising number of terminations of severe cases [3]. In addition, the morbidity of the survivors has increased, leaving them to deal with conditions such as chronic lung diseases, pulmonary hypertension, gastroesophageal reflux, oral feeding aversion, poor weight gain, hernia recurrence, hearing loss and delayed neurodevelopment [4]. Though modern medicine has always strived to find the origin and the perfect treatment for CDH and its consequences, the scientific and medical progress and the outcome has remained unsatisfactory, leaving many questions unanswered.

1.2. The development of the diaphragm in humans and rats

After the pleural cavities have separated themselves from the pericardial cavity, they still show a connection to the abdominal cavity, because the diaphragm is not fully formed yet. Caudal to the pleural cavity borders a crescent-shaped fold, known as the pleuroperitoneal fold (PPF). This fold starts to bulge out into the pericardioperitoneal canals, which lead into the abdominal cavity. By the seventh gestational week, the pleuroperitoneal plica fuses with the mesentery of the esophagus and the septum transversum. Thus, the connection between the thoracic part of the coelom and the abdominal cavity are separated by the pleuroperitoneal membrane. This membrane is enlarged by a peripheral band through the expansion of the pleural cavities. Finally, surrounding myoblasts are able to penetrate the membrane and so create the muscular part of the diaphragm [1]. The phrenic nerve spreads throughout the diaphragm, just after the muscle precursors arrived at the pleuroperitoneal membrane.

Though many theories about the formation of CDH exist, it is believed that the PPF plays an important role in this malformation. In human embryos the development of this PPF takes place between gestational weeks 4 to 6, at a crown to rump length of about 6 to 14mm. To find out, whether or not a defect in the PPF can directly lead to CDH, animal models have been investigated.

Rat embryos seem to have a similar development of the diaphragm, especially of the PPF, occurring between days 12 to 14 after insemination at a crown to rump length of approximately 5 to 9mm [5].

1.3. The development of the lungs

1.3.1. The embryonic period (0-5 weeks)

The development of the lungs starts with the formation of the sulcus laryngeotrachealis, which can be described as a channel in the ventral, lower pharynx. Its caudal ending later expands and builds the respiratory diverticulum [6,7].

1.3.2. The pseudoglandular period (5-16 weeks)

The lung's diverticulum constricts itself from the proenteron and meanwhile grows caudal. To the middle it forms the trachea and the lung buds are build lateral. The right lung bud divides into three branches and the left lung bud into two branches, presenting the grounds for the main bronchi and the pulmonary lobes.

The lung buds grow into the coelom cavity and thereby push the visceral mesoderm, which covers them, forward. In this area, the abdominal cavity is called the pericardioperitoneal canal or the coelom cavity. These canals communicate with the pericardial cavity above and the peritoneal cavity below. The closure of these openings the pericardioperitoneal canals develop into the pleural cavities. The visceral mesoderm becomes the visceral pleura and the parietal mesoderm the parietal pleura, forming the pleural cavity in between.

1.3.3. The canalicular period (16-26 weeks)

Further on, the main bronchi part again, building ten tertiary bronchi (segmental bronchi) in the right lung and eight or nine in the left lung. This definite formation correlates with the bronchopulmonal segments of an adult's lung. By the end of the sixth gestational month, about seventeen of these divisions have taken place, but six further divisions have yet to occur, before the bronchial tree reaches its ultimate shape. This branching happens after birth and is regulated by an interaction of epithelium and mesenchyme between the endoderm of the lung bud and the surrounding visceral mesoderm. The signals for the induction of the branching belong

to the family of the FGFs (fibroblast growth factors). With the continuing development, the lungs are relocated caudalward. At birth, the bifurcation of the trachea can be found aloft the fourth thoracic vertebra [1].

1.4. The maturation of the lungs

1.4.1. The saccular period (26 weeks-birth)

Until the seventh gestational month, the bronchial tree divides into smaller and smaller canaliculi and the density of the capillary network rises. However, the ability for proper respiration can only be guaranteed, once the cubic epithelium in the respiratory bronchioles differentiates into alveolar epithelial cells. These cells keep a close connection to capillaries and lymphatic vessels and thereby build a terminal branching, known as primary alveoli. From the seventh month on, enough alveoli and capillaries exist for the survival of a premature infant [1].

1.4.2. Alveolar differentiation (8 months-early childhood)

During the last two gestational months until several years after birth, the number of primary alveoli continues to grow. The alveolar epithelial cells become thinner, causing the capillaries to bulge into the primary alveoli. This close contact is known as the blood-air-barrier. Besides the thinned out alveolar epithelial cells (Type 1), Type 2 cells with a foaming cytoplasm differentiate inside the alveoli and produce Surfactant. Right before birth, the lungs contain a fluid with a high concentration of salt, some mucus from the bronchial glands and a rising amount of Surfactant from the alveolar epithelial cells Type 2. The fetal breathing movements start before birth, stimulate the lung's development, train the muscles of respiration and lead to an aspiration of amniotic fluid. Part of the fluid is squeezed out through the trachea by the process of birth and the rest is absorbed rapidly by the blood and lymphatic capillaries. Only a thin layer of surfactant remains on the alveolar epithelium, decreasing the surface tension and preventing atelectasis and the regurgitation of serous fluid into the alveoli during expiration.

Due to the onset of the breathing process after delivery, the lungs enlarge and fill out the pleural cavities. The growth of the lungs after birth can mainly be explained by the proliferation of respiratory bronchioles and alveoli and not so much by the

augmentation of the alveoli. At birth only a sixth of alveoli are formed, the main part develops during the first ten years of life through constant neoformation [1].

Lung development in rats also occurs in these phases described in human embryos and therefore appears to serve as an excellent model for research.

1.4.3. Pathogenesis of lung hypoplasia

Due to the abdominal organs that push through the CDH, the lung is compressed and left with little space for development and growth. The lung hypoplasia found in fetuses with CDH includes a failure of alveolar and pulmonary vascular development [4]. A reduction of branching decreases the number of airway generations and epithelial cell differentiation is believed to be delayed, because of an arrested development in the canalicular stage [2].

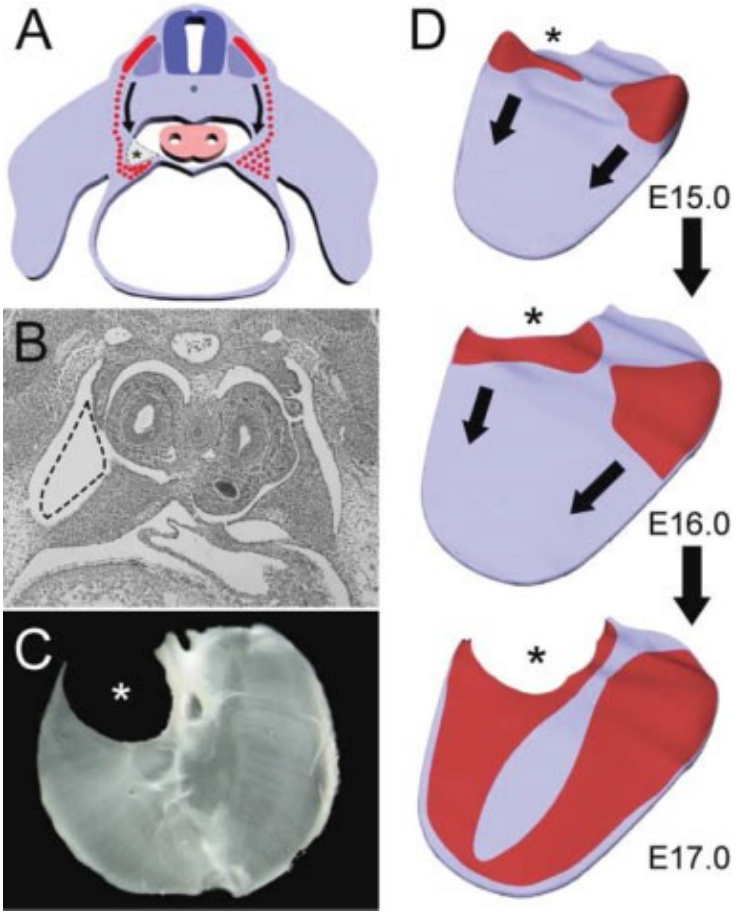
1.5. CDH nitrofen-model of the rat

The similarities between the normal and healthy development of the lung and the diaphragm in humans and rats also implicate analogue pathomechanisms in case of CDH. Therefore we used *Sprague-Dawley* rats for our study. In order to provoke diaphragmatic hernias in rat embryos, the mother has to be exposed to nitrofen. Originally, nitrofen was used as an herbicide and was adjudged to be relatively nontoxic to adult animals. However it has shown to have a teratogenic effect on the unborn rats, when administered to the pregnant dam. This mainly includes pulmonary, but also renal and cardiovascular malformations and complications. When nitrofen is administered on gestational day 9 or 11 after insemination, diaphragmatic hernias can be observed in the majority of cases. Interestingly, the administration on day 11 almost exclusively results in right-sided hernias, while a single dose of nitrofen on day 9 predominantly leads to left-sided hernias. We chose day 9 for the administration, in consequence all resulting hernias were left-sided ones. Nitrofen-induced CDH origins at a very early stage of lung development. It may differ in severity and location, similar to human CDH. In addition, as a result of the induced hernia, the phrenic nerve is reduced in diameter on the affected side [8].

Clugston et al. looked behind the cellular mechanisms of nitrofen-induced CDH and concentrated on the pleuroperitoneal fold as the central target of malformation. As already mentioned, the PPF of rats experiences a rapid development between

gestational days 12 to 14 after insemination. If nitrofen is applied, abnormalities of the PPF can first be observed between days 13 to 13.5, exactly falling into the period of major growth. Two hypotheses on how nitrofen induces CDH were tested, one suggesting decreased cell proliferation and the other one increased apoptotic processes. Murine fibroblasts were chosen as a proxy to mesenchymal cells of the developing diaphragm. Decreased cell proliferation was found to be the primary mechanism leading to an abnormal PPF and consequently to CDH. Apoptosis did not appear to be a crucial factor; however it might contribute to CDH when high doses of nitrofen are reached [5].

Keijzer et al. even suggest that nitrofen does not primarily induce diaphragmatic hernias and proposes CDH as a consequence of lung malformation. The lungs showed a reduced branching morphogenesis prior to the detection of the diaphragmatic defect [2].



(A) Schematic representation of a transverse cross-section through an E 13.5 rat embryo. Normally, muscle precursor cells (red) migrate to the triangular-shaped PPF, from which they later spread to populate the entire diaphragm.

In CDH, it is hypothesized that the PPF is malformed, which corresponds to a hole in the fully-formed diaphragm.

(B) Transverse section of a nitrofen-exposed E 13.5 rat embryo; the PPF on the left is malformed (dotted lines), compared with the right side of the body.

(C) Plan view of a diaphragm isolated from a nitrofen-exposed rat (E 16.5) showing a Bochdalek-type diaphragmatic hernia in the left posterolateral region of the diaphragm.

(D) Schematic representation of the developing diaphragm between approximately E 15 and 17. Red regions represent the population of muscle precursors that originate in the region of the PPF and spread to populate the entire diaphragm (arrows). An abnormality in the PPF thus translates to a hole in the fully formed diaphragm. [5]

Fig. 4

Embryogenesis of diaphragm defects in CDH

An explanation for the teratogenic effect of nitrofen could be its structural similarity with the thyroid hormone. It is assumed, that nitrofen is able to bind to the thyroid binding globulin (TBG) and thereby raise the level of free thyroid hormones. This leads to an increased thyreomimetic response [9].

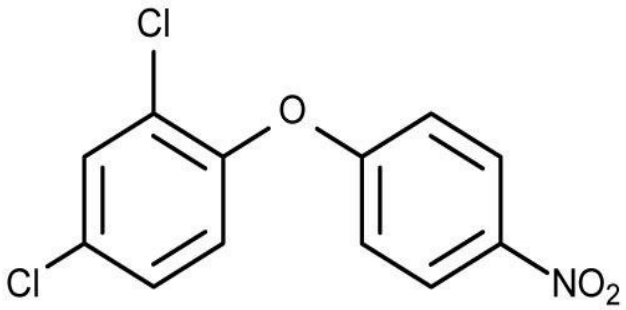


Fig. 5

Chemical structure of nitrofen

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Fig. 6

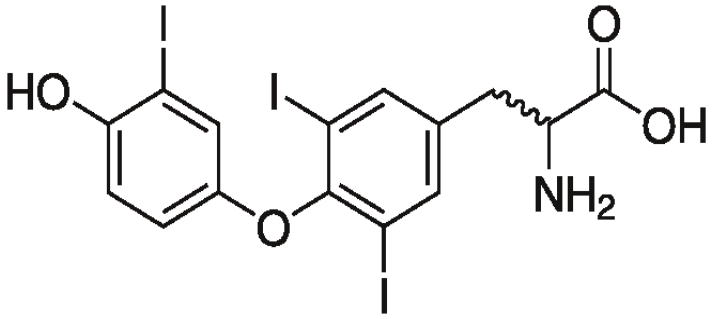

Fig. 7

Chemical structure of triiodothyronine (T3)

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Fig. 8

Another hypothesis comes from Ten Have-Opbroek et al., suggesting that nitrofen could actually interfere in the thyroid hormone metabolism. According to this study, a non-competitive inhibition of the nuclear triiodothyronine (T3) –receptor could trigger a thyreostatic effect [10].

1.6. The role of Insulin-like growth factors (IGFs)

Although the rat serves as an excellent model for CDH research, the exact pathomechanism of this condition has yet remained undetected. Still, humans with CDH and rats with nitrofen-induced CDH all exhibit the same major problem of lung

hypoplasia. As mentioned above, present treatments include prenatal surgery or postnatal stabilization with different ventilation and oxygenation techniques, respectively delayed surgery. But the search for less invasive prenatal treatments has increased interest on the effects of the family of insulin like growth factors (IGF).

IGFs are polypeptides with a sequence, which is very similar to that of proinsulin. This enables them to bind not only to their insulin-like growth factor receptor (IGFR), but also, with a lower affinity, to the Insulin receptor (IR). In return, Insulin can also bind to the IGFR. For their transportation in the blood stream, IGFs use binding proteins (IGFBP). IGFs are hormones and influence a number of functions throughout the body and are involved in fetal and placental developments through either paracrine or autocrine pathways. They can be sub-divided into IGF-1 and IGF-2.

IGF-1 is encoded on chromosome 12 and mainly produced by the liver. Its receptor (IGF1-R) can be found on almost all cells of the body and binds to IGF-1 with the highest affinity, but also to IGF-2 and with the lowest affinity to insulin. The hormone acts on the anabolic metabolism of carbohydrates and protein as well as cell proliferation. IGF-1 regulated growth is known to be the main mediator of insulin postnatal.

IGF-2 is located on chromosome 11 and can be formed by many bodily cells. It binds to IR or to the IGF1-R and of course to IGF2-R, also known as the Mannose-6-Phosphate receptor. This receptor only binds to IGF-2. IGF1-R conveys the information for growth and it is suggested that IGF2-R also serves as a clearance receptor for IGF-2. IGF-2 acts as a stimulator for growth, especially prenatally.

There are six types of IGFBP, which specifically bind to IGF-1 and -2 with high affinities. They usually limit the availability of the IGFs for the tissue. However some studies show that they might even promote the IGF's actions in some cases.

IGFs, particularly type 1, are involved in several processes of growth. They can provoke cell division of fibroblasts, myoblasts, osteoblasts, dermal and epithelial cells, gonadal cells and sometimes even tumor cells. IGF-1 can inhibit apoptosis in tumor cells and hematopoietic cells. It also stimulates cell differentiation in myoblasts, bone cells, neural cells and adipocytes. IGFs boost the production and the release of

hormones in ovarian cells and with the help of IGF-1, the number of adrenocorticotropic hormone (ACTH) receptors in the adrenal gland is increased [11].

Regarding pregnancy and fetal development, many theories of the role of IGFs have been investigated in the past years, answering many questions and bringing up plenty of new ones.

Bloomfield et al. investigated the fate of IGF-1, when administered into the amniotic fluid of fetal sheep. They found, that the injected IGF-1 quickly distributed in the amniotic fluid and was then swallowed by the fetus. Therefore it could reach the intestinal organs and furthermore, enter the portal venous circulation. IGF-1 remained detectable in the amniotic fluid for six more days in bound and free forms [12].

Bastian et al. discovered the transport of IGF-1 across epithelial cell monolayers, found in the gut, in kidneys and the lung. They reported a restricted diffusion of free IGF-1 across these monolayers and suggest a “paracellular or low-affinity transcellular pathway” instead of a receptor-mediated transport [13].

Eremia et al. could show that the administration of IGF-1 into the amniotic fluid actually improves the outcome of fetal sheep suffering from intrauterine growth restriction (IUGR) [14]. Another study by Skarsgard et al. reports the same results in a similar experiment with fetal rabbits [15]. Tarantal et al. administered IGF-1 intraperitoneal to fetal rhesus monkeys and observed increased organ weights and sizes of the thymus, the spleen, the kidneys and the small intestine. This confirms the importance of IGF-1 as a growth promoter during fetal development [16]. In addition, IGF-1 also acts on the mother’s body composition during pregnancy, as Roberts et al. proved by the help of guinea pigs. Since IGF-1 is not produced abundantly by the placenta, it is believed that IGF-1, predominantly coming from the mother, acts on the IGF1-R located in the placenta and thus spreading the effects on the fetus [17]. According to Sferuzzi et al., IGF-1 does not have an effect on the placenta, but it helps to reduce adiposity of the guinea pig mother in late pregnancy [18].

Sibley et al., Regnault et al. and Constancia et al. all state that IGF-2 plays an important role in the regulation of the diffusional permeability of the mouse placenta and may therefore be crucial for the transport of maternal nutrients and consequently the growth of the fetus [19,20,21]. Sferruzzi et al. show that IGF-2 administration to

the mother subcutaneously in early or mid- pregnancy increases the volume and the surface area of the placenta, enabling a better supply for the guinea pig fetus [18]. IGF-2 may also have an influence on the blood supply to the placenta and on its overall differentiation. These effects are mostly mediated by the IGF2-R, but the IGF1-R might also play a participating role. This suggests that an IGF-2 deficiency may cause an intrauterine growth restriction [17].

Both, IGF-1 and IGF-2 seem to be involved in fetal and placental growth and development. They prevent fetal absorption and cause the circulating concentrations of amino acids in the fetus to rise [18]. This suggests that treatment with IGFs may also be a possible therapy for human fetuses with growth restriction.

As regards of the role of IGFs in embryonic lung development, both types of IGFs are evenly expressed throughout the mesenchymal tissue. However, IGF-2 mRNA can mainly be found in epithelia. At the beginning the expression of the IGF1-R is pervasive, but later in development it becomes limited to the mesenchyme. The IGF2-R is located exclusively in the mesenchyme and some intrapulmonary vessels. In the embryonic lung, IGFBP 2, 4 and 5 are prevalent, forming a regulatory system throughout the lung. Type 2 is mainly expressed in epithelial cells and the mesenchyme, such as Type 4. On the contrary, Type 5 expression increases over time and is located in the epithelial lining of the terminal bronchi [22].

Silva et al. investigated the influence of IGF-2 on the fetal development of the lung. They used mice with a null mutation of IGF-2 and observed the impact on the lungs. They reported a deferred lung development and differentiation, which might be due to low maternal corticosterone levels. In addition, the expression of surfactant protein B (SP-B) and IGF-1 mRNA was altered. This leads to the conclusion, that IGF-2 interacts with many different endocrine systems and its deficiency causes an imbalance of multiple processes in the maternal and fetal body [23].

Nevertheless the actions of the IGFs in combination with their receptors can sometimes create unwanted results. There is evidence that IGF-1 could play a role in the formation of fibrosis in the lungs. Elevated levels have been found in the bronchoalveolar lavage fluid of patients suffering from acute respiratory distress syndrome (ARDS). Choi et al. blocked the IGF1-R with an antibody in mice treated

with bleomycin, a substance causing lung injury and fibrosis. The blockade let to an increased apoptosis of fibroblasts, eventually resolving the fibrosis [24].

IGFs also seem to be involved in the embryonic development of the diaphragm. Fournier et al. performed a gene disruption of IGF-1 in mice and evaluated the impact on the diaphragm. The gene deletion resulted in a decreased number of muscle fibers, a muscular hypoplasia, and the capillary network was reduced. The muscle fibers showed a retardation of growth and the size of the cells was smaller compared to healthy mice diaphragms. These findings demonstrate the importance of IGF-1 in the developmental mechanisms of the diaphragm. They suggest that IGF-1 influences cell proliferation and differentiation and may also be involved in programmed cell death (apoptosis). Interestingly, the oxidative capacity of the muscle fibers proved to be intensified, which appears to be a process of adaptation, thus ameliorating the chances of survival [25].

Nagata et al. also wanted to get an insight on the roles of IGFs regarding lung development in order to examine their authority as a possible treatment for the hypoplastic lungs of CDH patients. The IGFs were found to play a decisive role especially in the late stages of lung development, as the number of IGF receptors increased as well. This indicates that IGFs could be considered as a possible treatment for CDH patients, helping their lungs to mature [26].

As the role of IGFs in healthy lung development was largely discovered, Esumi et al. prepared for the next step. They actually tested if IGFs represent a possible therapy for CDH patients. Pregnant rats were exposed to nitrofen, resulting in a CDH of the offspring. Fetuses were harvested early and the lung specimens were kept in cultures of IGF-1, IGF-2 or control mediums. Surprisingly, only lungs grown in the IGF-2 medium demonstrated an improved alveolar maturation. Esumi et al. therefore proposed a prenatal IGF-2 treatment for patients with lung hypoplasia due to CDH [27].

1.7. Reference to this trial

In our study, we performed a comparable scientific trial, with the difference of treating the fetal rats in vivo. We injected IGF-1, IGF-2 or saline solution intraamniotically in pregnant Sprague-Dawley rats with the nitrofen induced CDH. A control group

remained untreated. The lungs of the offspring were harvested prior to natural birth and stained with different markers for immunohistochemistry. The applied primary antibodies included Ttf-1 (Thyroid Transcription Factor-1), Pdpn (Podoplanin), α-SMA (Alpha-Smooth Muscle Actin), Fgf10 (Fibroblast Growth Factor-10) and Ki67 (MKI67).

1.8. Antibodies used in immunohistochemistry

Ttf-1, amongst other functions, influences the production of surfactant in the lungs and thus has an impact on the alveolar epithelial cells Type 2. Therefore we took interest in comparing its expression in the lungs of our different trial groups.

Pdpn, also known as T1- α, seems to be involved in the development of the lung and in the regulation of lymphatic vascular formation. It served as a marker for the alveolar epithelial cells Type 1.

Immunohistochemistry using α-SMA helped portray and evaluate the smooth muscle found in the bronchia and vessels of the lung.

Fgf-10 is assumed to play a role in the development of lung buds and can therefore be used as a marker to determine the quantifiable alteration of its expression in the IGF treated lungs.

Ki67 served as a marker for unspecific cell proliferation, as it is a nuclear protein which is expressed in all active parts of the cell cycle. Also in rats, it can be used to analyze the growth rate of a population of cells. Ki67 therefore helped illustrating the changes in proliferative activity in the fetal lung cells after the application of the IGFs.

1.9. The aim of the study

We wanted to investigate if intraamniotically applied IGF-1 or IGF-2 has a beneficial effect on the differentiation or proliferation of hypoplastic lung tissue of fetal rats.

We hoped that our approach would approximate a realistic therapeutic process and the results would be valuable for future treatments of hypoplastic lungs in humans.

2. Materials and methods

2.1. The practical bioessay

The aim of this experimental study was to investigate the effect of intraamniotically injected recombinant human IGF-1 (rhIGF-1) and rhIGF-2 on hypoplastic lungs of fetal rats with nitrofen-induced congenital diaphragmatic hernia (CDH).Time dated pregnant *Sprague–Dawley* rats were treated with nitrofen to cause CDH and lung hypoplasia in the offspring.

Adult rats were mated and the presence of spermatozoids in the vaginal smear, which confirmed pregnancy, was considered gestational day 0 (E0).

The intra-amniotic application of rhIGF-1, rhIGF-2 and nitrofen treatment were performed on these pregnant female rats, which were obtained from the University of Vienna, Core Unit for Biomedical Research, Division for Laboratory Animal Science and Genetics, 2325 Himberg.

All surgical interventions as well as the application of nitrofen were carried out at the Institute for Biomedical Research at the Medical University of,Graz, where the rats were housed. The animal experiments were approved by the Federal Ministry of Science and Research, Austria (GZ: BMWF-66.010/0011-II/10b/2010).

2.1.1. The administration of nitrofen

The administration of nitrofen to the pregnant mother rats was conducted under short time anaesthesia by isofluran and oxygen, after the calculation of their individual body weights. On E9.5 1000mg of nitrofen (2,4-dichloro-1(4-nitrophenoxy)benzene, ACD-Code: MFCD00128026; Maybridge, Thermo Fisher Scientific) were dissolved in 10 ml of olive oil, afterwards 1ml of this solution was applied to each mother rat via stomach tube (equal 100 mg nitrofen per dam).

The correct placement of the stomach tube was checked prior to the administration of the solution by the help of a scaling on the tube (this way, an accidental application to the airways could be ruled out). After the administration of nitrofen, the rats were brought back into their cages, put in a lateral position and observed for another 30 minutes in order to identify complications like aspirations as soon as possible.

2.1.2. Surgical interventions and rhIGF-injections

For the surgery on E19 and 20, the rats were weighed and anaesthetized in an induction box by 4% isofluran (Forane®, Abbott, Austria) in oxygen by using a vaporizer (TEC3®, Omnisa, Switzerland).

Afterwards, placed on a heating pad (37°C), anaesthesia was continued by isofluran treatment with a tube connected to mouth and nose of the rat and to the vaporizer (1.5-2.5% isofluran in 100% oxygen).

The rats were locally anaesthetized by a subcutaneous injection of lidocain (Xylanest purum® 2%, Gebro Pharma, Austria) directly into the area of surgery, applying a dosage of 1µg/mg body weight. A subcutaneous injection of tramadolhydrochlorid in a dosage of 15µg/mg body weight (Tramabene®, Ratiopharm, Germany) was chosen as pain relief therapy. Carprofen, injected subcutaneously (Rimadyl®, Pfizer, Austria) in a dosage of 5µg/mg body weight, was used as an anti-inflammatory treatment. Another subcutaneous injection of enrofloxacin (Baytril® 2.5%, Bayer, Austria) in a dosage of 7.5µg/mg body weight covered the antibiotic therapy.

Under sterile conditions the abdomens of the rats were opened and the wombs were exposed. Counting from the vaginal to the ovarian end, the number of fetuses was documented in both uterine horns. The animals were divided into four groups: IGF-1-, IGF-2-, Placebo-, and Control group. In each case 0.2µg of rhIGF-1 (10µg, No. Z00362; GenScript Inc., USA) dissolved in 50µl sterile saline (IGF-1 group), 0.2µg of rhIGF-2 (10 µg, No. 130-093-888; Miltenyi Biotec Inc., USA) dissolved in 50µl sterile saline (IGF-2 group) or 50µl saline only (Placebo group) were injected into the amniotic fluid by using a syringe.

The rats of the control group remained without any intervention and served the establishment of a „baseline“.

The application of rhIGF-1 apparently affects lung development according to studies of Eremia et al. (in fetal sheep, in vivo) and studies of Nagata et al. (in lung cell cultures of mice, in vitro). The dosage of rhIGF-1 (0.2µg, i.e. 0.06µg/g weight on E19 and 20) was chosen referring to those studies. The weight of a rat fetus on the day of the intra-amniotic injection (E19 and 20) averaged about 2.5 to 3g.

The surgery ended by closing up the fascia abdominalis and the skin by sutures (Vicryl rapide®, Ethicon®, 3-0, 70 cm, FS-1; Johnson & Johnson, Germany). On E20, the intra-amniotic injections were repeated by removing the sutures and replacing them after the intervention. On E21.5 all of the fetuses were taken by caesarean section and afterwards the mothers were euthanized by cardiotomy under deep anaesthesia.

The fetuses were weighed and fetal necropsy was performed. Fetal left lungs were taken, weighed and shock frozen in liquid nitrogen. Only vital fetuses, which showed a left-sided CDH on E21.5 were used for this study.

2.1.3. Humane closure (criteria that indicated euthanasia or the abruption of this experiment)

In case of an inadequate weight gain of an operated, food refusing mother rat, the occurrence of serious respiratory complications of the mother rat or if noticeable lethargy (especially after the application of nitrofen or during the first 24 hours after the individual surgery) could be observed, the experiment was discontinued. This also applied to rats that could not be impregnated.

2.2. Practical Immunohistochemistry

In this study, the left lungs of fetal rats with CDH have been selected for the immunohistochemical staining.
After the fetal rats‘ left lungs had been removed surgically, they were transferred into Cryo-tubes (Cryo.S®, Cellstar®; Greiner Bio-One GmbH) and shock-frozen in liquid nitrogen. The samples then remained stored until the creation of tissue samples.

By means of the Cryostat (HM 560 Cryo-Stat®; Microm GmbH), tissue slices of 7µm were cut (embedding: O.C.T.-Compound, Tissue-Tec®; Sakura, USA) and put on slides (Super Frost®Plus; Menzel GmbH) to dry at room temperature overnight. Afterwards, the samples were stored at -20°C.

The tissue slices for the staining for Ttf-1, PDPN, SMA, Fgf10 and Ki67 were fixed in 100% acetone (No. 100014, Merck, Austria) for 10 minutes at room temperature. The

next step was the dehydration at room temperature and then the tissue slices were outlined with a special water-repellent pen (DAKO-Pen®, No. S 2002; DAKO GmbH).

For rehydration, the samples for SMA were put in PBS for 10 minutes (pH 7.2-7.3; Na2HPO4: 14.625 g, No. 6580, Merck, Austria; KH2PO4: 2.45 g, No. 4873, Merck, Austria; NaCl: 80.0 g, No. 6404, Merck, Austria; sterile Aqua dest.) and the ones for Ki67, Ttf-1, PDPN, and Fgf10 in TBS (Tris Base, No. 8382, Merck, Austria; NaCl, No. 6404, Merck, Austria; Aqua dest.) according to the protocol of IHC-World: http://www.ihcworld.com/_protocols/washing_buffers/pbs.htm).
Afterwards the peroxidase-blockage was carried out through the treatment of the tissue samples (SMA, Ttf-1, PDPN, and Fgf10) with 0.3% H2O2 (hydrogen-peroxide, 30%, No. 95313, Fluka GmbH) and methanol (Methanol pure, No. 6009, Merck, Austria) for 30 minutes each.
After the purging, the block-step was conducted for 60 minutes with HorseN-Serum (10%, No. sc-2483, Szabo Scandic, Austria) and BSA (1%, No. A7906, Sigma Aldrich, USA) in PBS (for SMA, Ttf-1 and PDPN); with 10% HorseN-Serum, 1% BSA and 0.5% Tween (Tween®20; No. 822185, Merck, Austria) in TBS (for Ki67); with GoatN-Serum (10%, No. X0907, DAKO GmbH), 1% BSA in TBS (for FGF10).

The staining with the primary antibody (1-3 hours) was arranged after the tilting:

Ttf-1: mouse monoclonal IgG 1:50, ab72876 (Abcam, UK), in Diluent (Anti-Body Diluent, No. S0809; DAKO, Austria);
PDPN: Anti-Rat Podoplanin Monoclonal Antibody, 1:200, Cat. No. 11-035 (EUBIO), in Diluent (DAKO, Austria);
SMA: Monoclonal Mouse Anti-Human Smooth Muscle Actin; 1:100, M0851 (DAKO, Austria), in Diluent (DAKO, Austria);
Ki67: Monoclonal Mouse Anti-Rat Ki67 Antigen; M7248; 1:200 (DAKO, Austria), in Diluent (DAKO, Austria);
and Fgf10: Santa Cruz FGF10 (H-121); sc-7917, 1:50 (Santa Cruz Biotechnology), in Diluent (DAKO, Austria).

After washing the tissue slices intensively, they were incubated with the secondary antibody (30 min):

Ttf-1, PDPN, SMA and Ki67: with BA 2001 (biotinylated Anti-Mouse IgG (H+L), produced in horse; Vector Laboratories), 1:200, in PBS;
Fgf10: with BA 1000 (biotinylated Anti-Mouse IgG (H+L), produced in goat; Vector Laboratories), 1:600, in PBS.
After the washing in TBS, respectively PBS the incubation of the tissue slices with DAB (Dako Liquid DAB+ Substrate Chromogen System; No. K3467; Dako, Austria) took place. 1 ml DAB substrate buffer and a drip of DAB Chromogen were added. Ki67 and Fgf10 were each stained for 30 minutes, Ttf-1 for 10 minutes, Podoplanin for 3 minutes and SMA for 1 minute. Afterwards the counterstain with the Hämalaun-solution (Mayer´s Hämalaun-Lösung, No. 9249; Merck, Austria) and the covering with glycerine-gelatine (Kaiser's Glyceringelatine, No. 9242; Merck, Austria), that had been pre-heated in the water-bath, followed.

The analysis of the stained tissue samples was conducted with the aid of the Aperio-system.

The software GraphPad Prism (Ver. 5.04; GraphPad Software Inc.) was used for the statistical interpretation of the gained data.

2.3. Analysis of tissue sections

The tissue sections of the left lungs were scanned by Aperio Scan Scope T3 (Scan Scope Console Version 7.00.08.1020, Controller Version 7.0.0.1024; © Aperio Technologies).

The quantitative analysis of the scanned pictures was performed by Image Scope (Version 10.1.3.2028; Image Nav 10.01.2028, Viewport 10.1.7/10.1.80; © Aperio Technologies).

The entire tissue sections were counted into three intensity bins by Positive Pixel Count (Version 8.100).

The following settings of algorithm inputs were used: View width: 1000; View height: 1000; Overlap size: 0; Image Zoom: 1; Classifier: none. Intensity Threshold weak: 220 (upper limit); Intensity Threshold weak: 175 (lower limit); Intensity Threshold medium: 175 (upper limit); Intensity Threshold medium: 100 (lower limit); Intensity Threshold strong: 100 (upper limit); Intensity Threshold strong: 0 (lower limit).

The number of strong positive cells (Nsp) and the total number of cells (NTotal) were counted and the percentage of strong positive cells was used for statistical analysis.

2.4. Statistical analysis

All data are presented as mean ± standard deviation (SD). Differences between IGF-1 or IGF-2 and the respective placebo group were tested by using the unpaired Student's t-test when the data were normally distributed. Mann–Whitney´s test was used when the data deviated from normal distribution. The values of the fetuses of the untreated control group served as a baseline. Statistical significance was accepted at p-values <0.05.

The statistical analysis of data was performed by using GraphPad Prism (version 5.04; GraphPad Software Inc.).

3. Results of the immunohistochemical staining

For the immunohistochemical analysis, the left lungs of the CDH positive fetal rats in the IGF-1 (n=10), IGF-2 (n=10) and the respective placebo groups (n=5 per group) were used. The left lungs of the fetal rats in the untreated control group (n=5), served as a baseline comparison (dotted line in each diagram).

The percentage of positive cells for each of the five different stainings was determined by Aperio Scan Scope.

3.1. Ttf-1-Staining

By analyzing the percentage of Ttf-1 positive cells in the left lung tissue, the productive capacity of Surfactant and furthermore the number and efficiency of the alveolar epithelial cells Type 2 can be evaluated directly.

The percentage of Ttf-1 positive cells summed up to **11.13** ± 8.74 in the IGF-1 group and **10.84** ± 7.89 in the placebo group of IGF-1 study, **0.78** ± 1.26 in the IGF-2 group and **3.60** ± 5.14 in the placebo group of IGF-2 study and **15.23** ± 7.49 in controls.

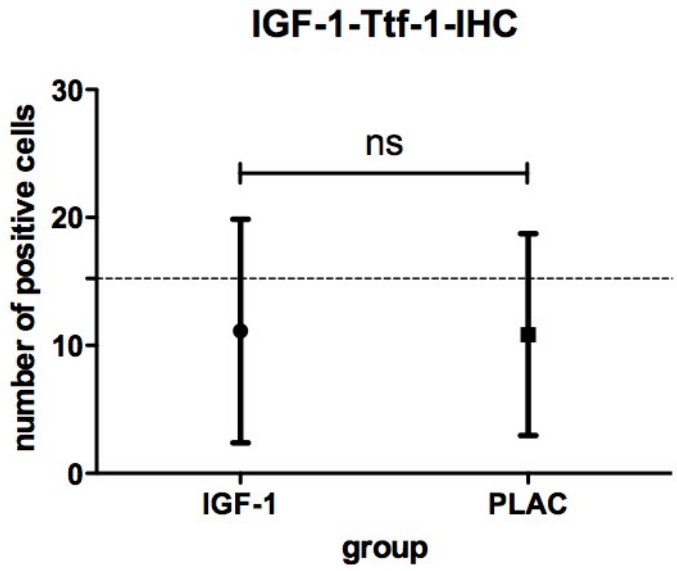

Fig. 4a: Ttf-1 expression: Results of the student´s t-test between IGF-1 and the associated placebo group.

The dotted line in every graph of the t-test shows the value of the untreated control group, which served as a baseline and was not used for statistical analysis. Values are shown as mean ± sd.

Fig. 4a

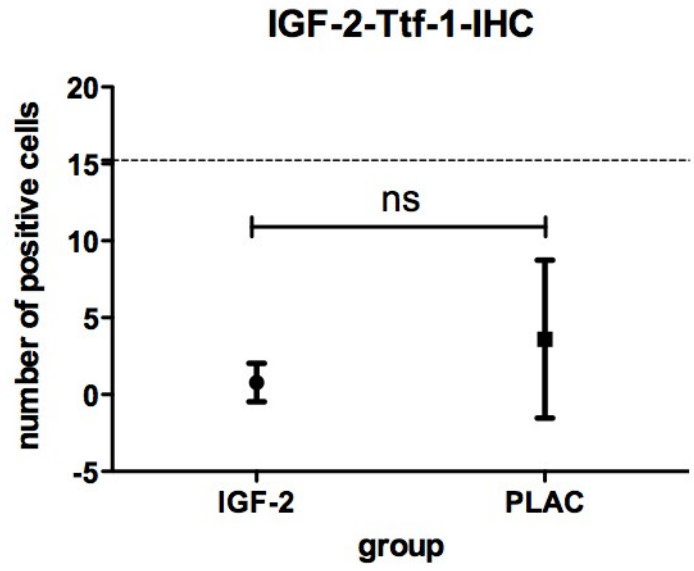

Fig. 4b: Ttf-1 expression: Results of the student´s t-test between IGF-2 and the associated placebo group.

The dotted line in every graph of the t-test shows the value of the untreated control group, which served as a baseline and was not used for statistical analysis. Values are shown as mean ± sd.

Fig. 4 a

The unpaired t-test between the placebo group and the IGF-1 group shows a non-significant difference between the percentages of Ttf-1 positive cells with a p-value of 0.9512 and a non-significant difference with a p-value of 0.2544 is determined between the IGF-2 and the placebo group.

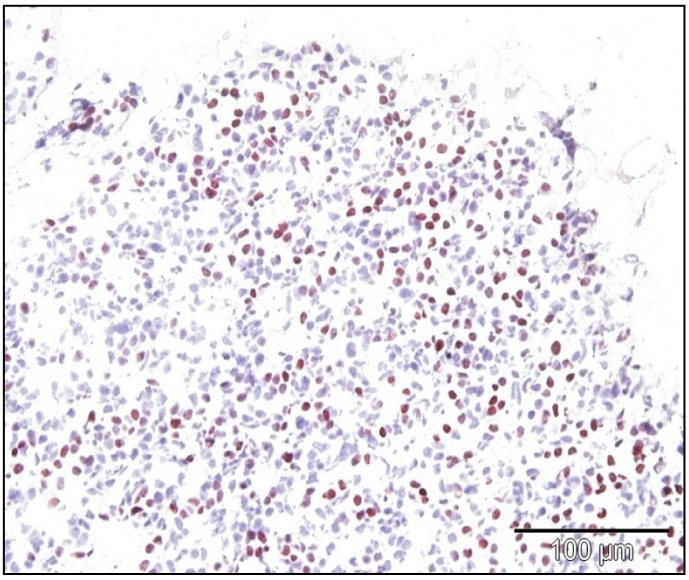

Fig. 4 b

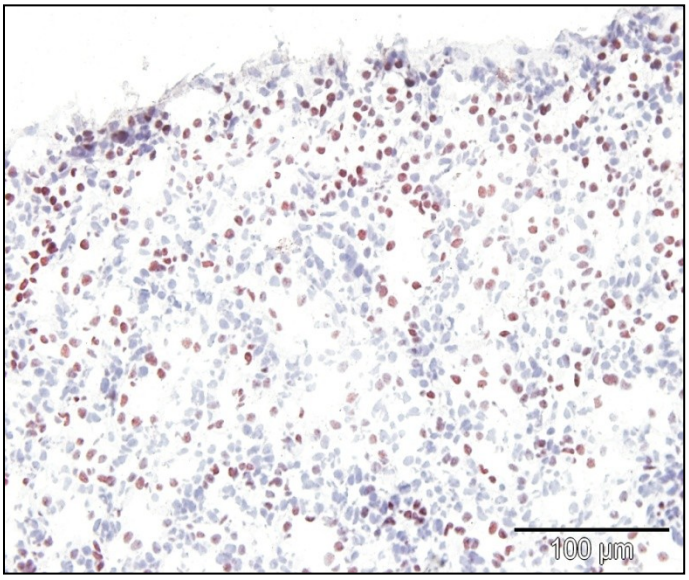

Fig. 4 c

Fig. 4 (c-d): Microscopic images of the **Ttf-1** immunohistochemical staining.

Upper image (Fig. 4c): **Placebo group**;

Lower image (Fig. 4d): **IGF-2 group**;

Magnification: 400-times; barr: 100µm

3.2. Pdpn/T1α-Staining

Pdpn serves as a marker for the alveolar epithelial cells Type 1.

The counting of the Pdpn-positive cells in the tissue slices shows mean percentages of **27.89** ± 14.05 in the IGF-1 group and **25.07** ± 6.73 in the placebo group of IGF-1 study, **11.05** ± 5.59 in the IGF-2 group and **8.69** ± 7.97 in the placebo group of IGF-2 study and **21.02** ± 8.94 in the control group.

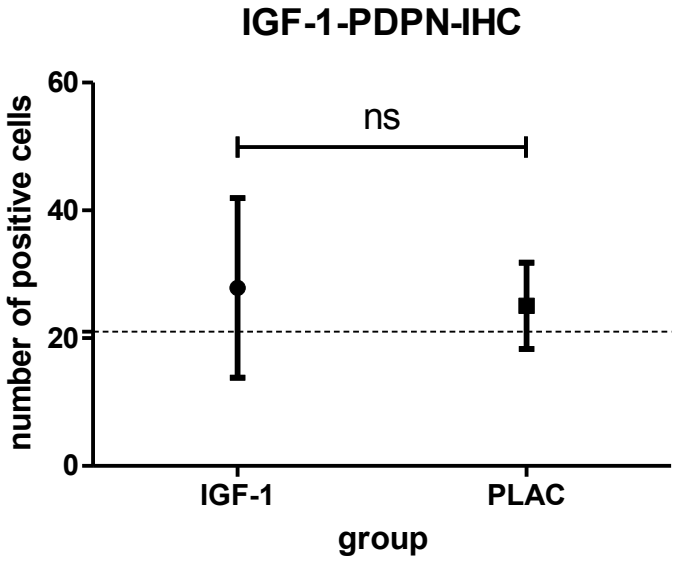

Fig. 5a: Pdpn expression: Results of the student´s t-test between IGF-1 and the associated placebo group.

The dotted line in every graph of the t-test shows the value of the untreated control group, which served as a baseline and was not used for statistical analysis. Values are shown as mean ± sd.

Fig. 5 a

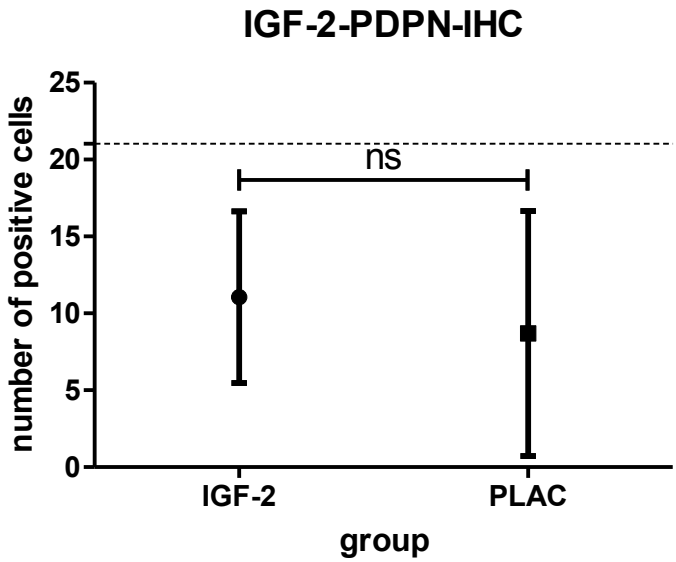

Fig. 5b: Pdpn expression: Results of the student´s t-test between IGF-2 and the associated placebo group.

The dotted line in every graph of the t-test shows the value of the untreated control group, which served as a baseline and was not used for statistical analysis. Values are shown as mean ± sd.

Fig. 5 b

The unpaired t-test did not portray a significant difference in the percentage of Pdpn-positive cells, neither between the placebo group and the IGF-1 group (p-value: 0.6814) nor between the placebo group and the IGF-2 group (p-value: 0.5141).

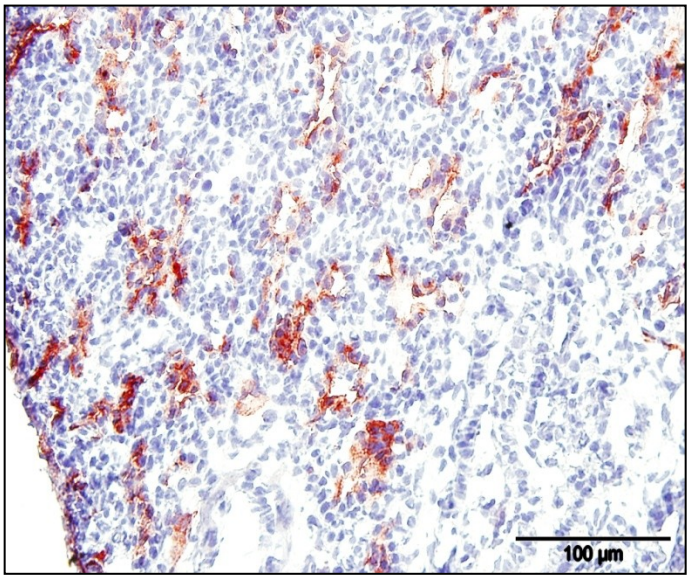

Fig. 5 c

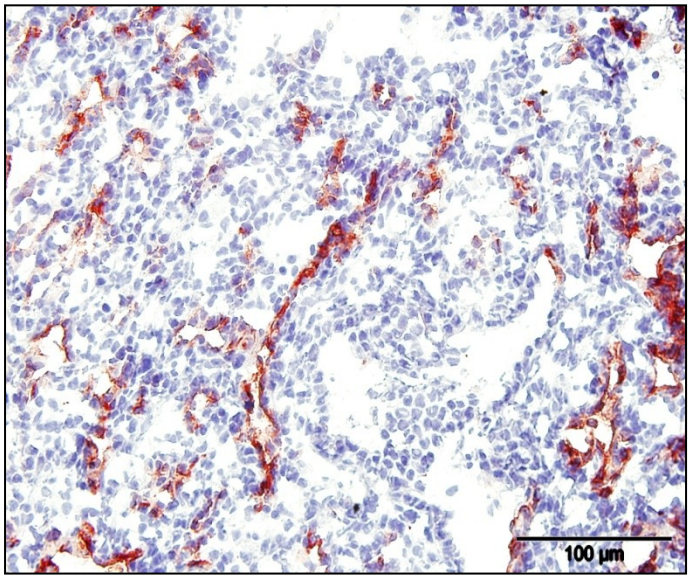

Fig. 5 d

Fig. 5 (c-d): Microscopic images of the **Pdpn** immunohistochemical staining.

Upper image (Fig. 5c): **Placebo group**;

Lower image (Fig. 5d): **IGF-1 group**;

Magnification: 400-times; barr: 100µm

3.3. SMA-Staining

Alpha-Actin-2, also known as (alpha) smooth muscle actin (α-SMA/SMA) is a protein, which is encoded in the ACTA2-gene in humans. In this study it was used to represent and evaluate the muscle in the bronchi and in the vessels of the lung.

On average, percentage of SMA-positive cells was **13.60** ± 6.36 in the IGF-1 group and **22.99** ± 10.69 in the placebo group of IGF-1 study, **5.13** ± 5.98 in the IGF-2 group and **6.05** ± 3.79 in the placebo group of IGF-2 study and **11.61** ± 7.37 in the control group.

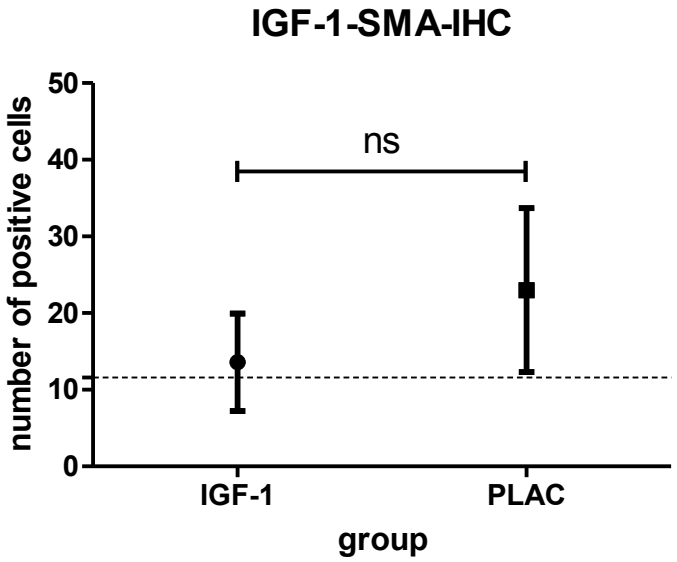

Fig. 6a: SMA expression: Results of the student´s t-test between IGF-1 and the associated placebo group.

The dotted line in every graph of the t-test shows the value of the untreated control group, which served as a baseline and was not used for statistical analysis. Values are shown as mean ± sd.

Fig. 6 a

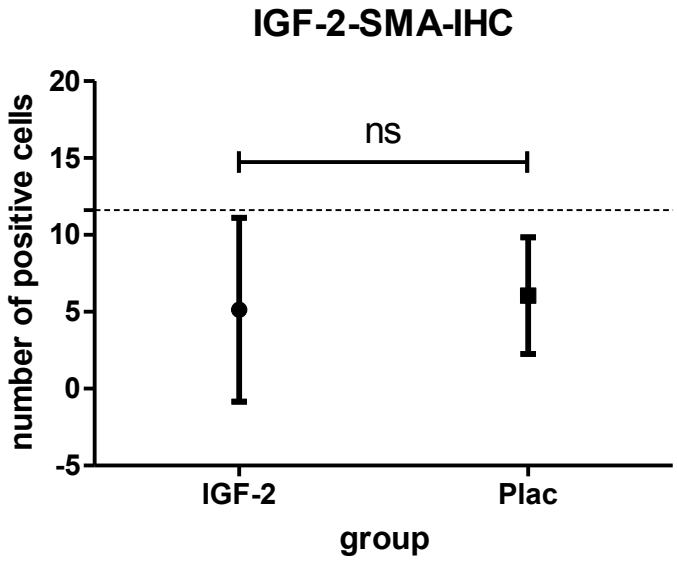

Fig. 6b: SMA expression: Results of the student´s t-test between IGF-2 and the associated placebo group.

The dotted line in every graph of the t-test shows the value of the untreated control group, which served as a baseline and was not used for statistical analysis. Values are shown as mean ± sd.

Fig. 6 b

The results of the unpaired t-test show no significant difference between the placebo group and the IGF-1 group (p-value: 0.0501) and a non-significant difference with a p-value of 0.7604 is determined between the IGF-2 and the placebo group.

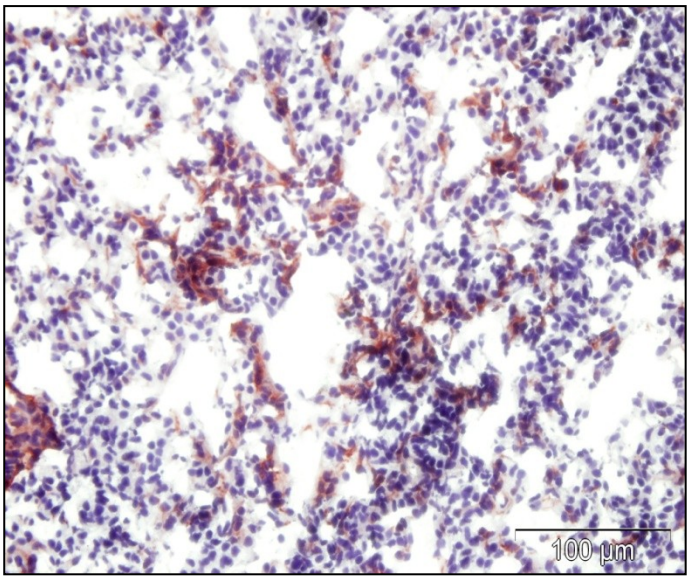

Fig. 6 c

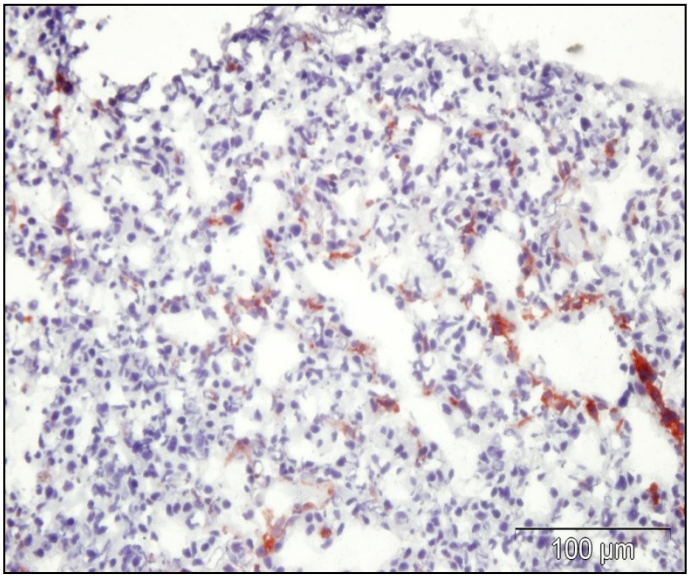

Fig. 6 d

Fig. 6 (c-d): Microscopic images of the **SMA** immunohistochemical staining.

Upper image (Fig. 6c): **Placebo group**;

Lower image (Fig. 6d): **IGF-2 group**;

Magnification: 400-times; barr: 100µm

3.4. Fgf10-Staining

The immunohistochemical staining with Fgf10 was conducted to examine the building of the connective tissue and the budding of the fetal rat lungs.

The average results of the Fgf10-positive cells in the lung tissue samples added up to **20.77** ± 8.11 in the IGF-1 group and **21.00** ± 13.03 in the placebo group of IGF-1 study, **0.39** ± 0.41 in the IGF-2 group and **3.59** ± 6.42 in the placebo group of IGF-2 study and **9.14** ± 12.30 in the control group.

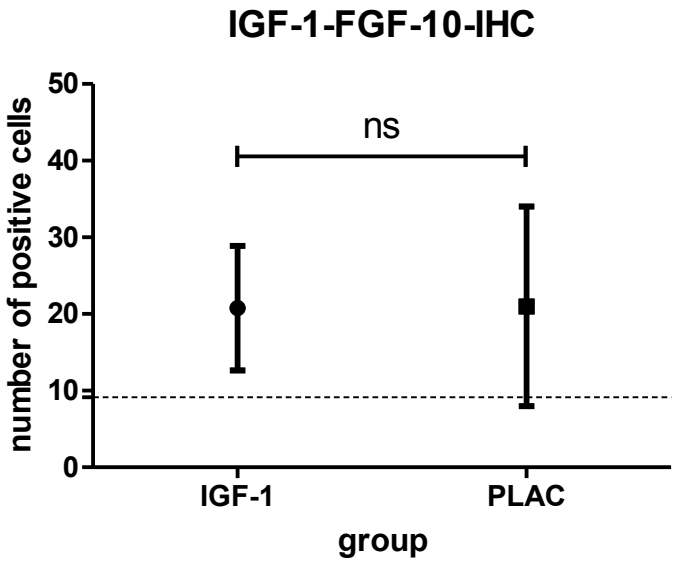

Fig. 7a: Fgf10 expression: Results of the student´s t-test between IGF-1 and the associated placebo group.

The dotted line in every graph of the t-test shows the value of the untreated control group, which served as a baseline and was not used for statistical analysis. Values are shown as mean ± sd.

Fig. 7 a

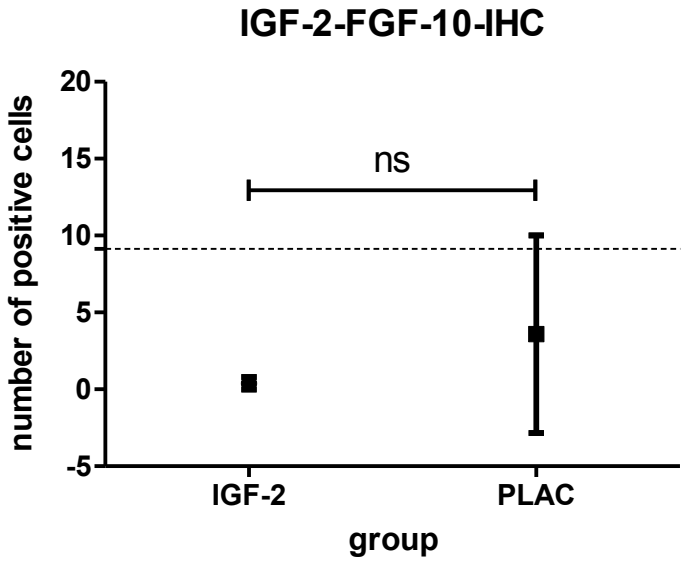

Fig. 7b: Fgf10 expression: Results of the student´s t-test between IGF-2 and the associated placebo group.

The dotted line in every graph of the t-test shows the value of the untreated control group, which served as a baseline and was not used for statistical analysis. Values are shown as mean ± sd.

Fig. 7 b

The unpaired t-test between the placebo group and the IGF-1 group shows a non-significant difference between the percentages of Fgf10-positive cells with a p-value of 0.9672 and a non-significant difference with a p-value of 0.1270 is determined between the IGF-2 and the placebo group.

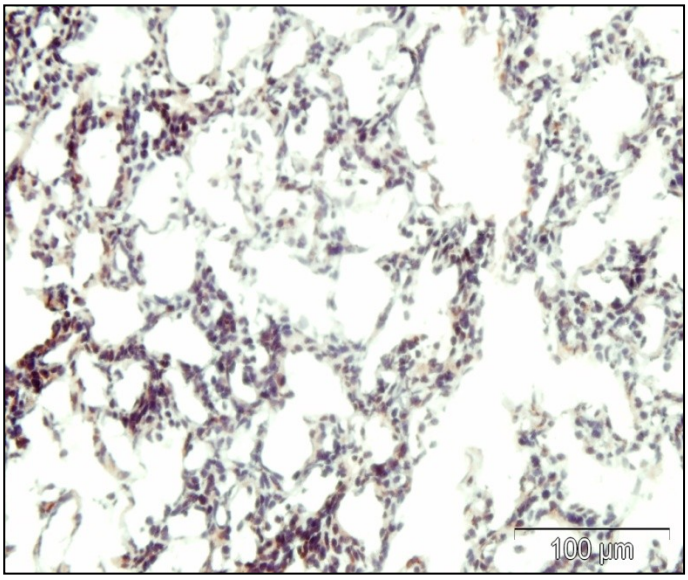

Fig. 7 c

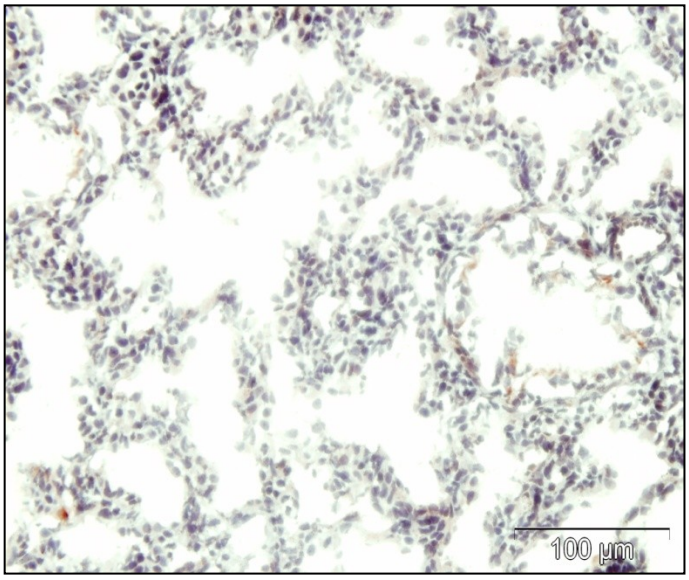

Fig. 7 d

Fig. 7 (c-d): Microscopic images of the **Fgf-10** immunohistochemical staining.

Upper image (Fig. 7c): **Placebo group**;

Lower image (Fig. 7d): **IGF-2 group**;

Magnification: 400-times; barr: 100µm

3.5. Ki67-Staining

Ki67 served as a marker to illustrate the general proliferative activity in the lung tissues.

The average values for the Ki67-positive cells in the fetal lungs sum up to **16.64** ± 12.13 in the IGF-1 group and **12.34** ± 9.50 in the placebo group of IGF-1 study, **0.39** ± 0.24 in the IGF-2 group and **1.33** ± 2.56 in the placebo group of IGF-2 study and **7.92** ± 3.72 in the control group.

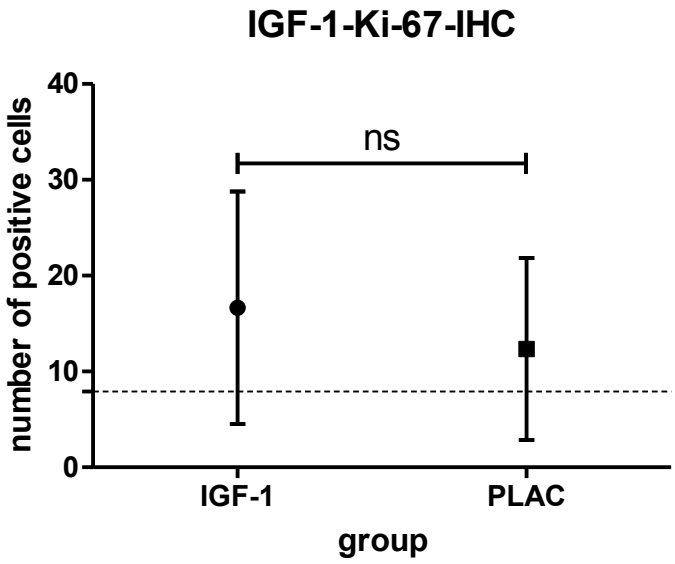

Fig. 8a: Ki67 expression: Results of the student´s t-test between IGF-1 and the associated placebo group.

The dotted line in every graph of the t-test shows the value of the untreated control group, which served as a baseline and was not used for statistical analysis. Values are shown as mean ± sd.

Fig. 8 a

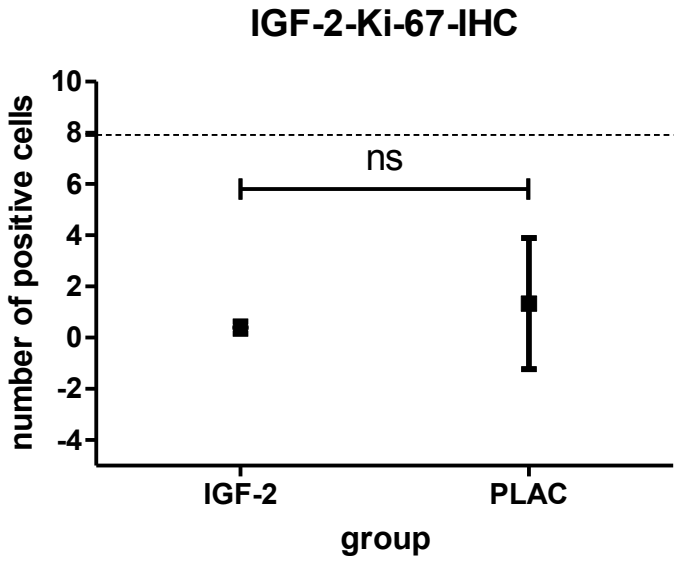

Fig. 8b: Ki67 expression: Results of the student´s t-test between IGF-2 and the associated placebo group.

The dotted line in every graph of the t-test shows the value of the untreated control group, which served as a baseline and was not used for statistical analysis. Values are shown as mean ± sd.

Fig. 8 b

The unpaired t-test between the placebo group and the IGF-1 group shows a non-significant difference between the percentages of Ki67-positive cells with a p-value of 0.5034 and a non-significant difference with a p-value of 0.2565 is determined between the IGF-2 and the placebo group.

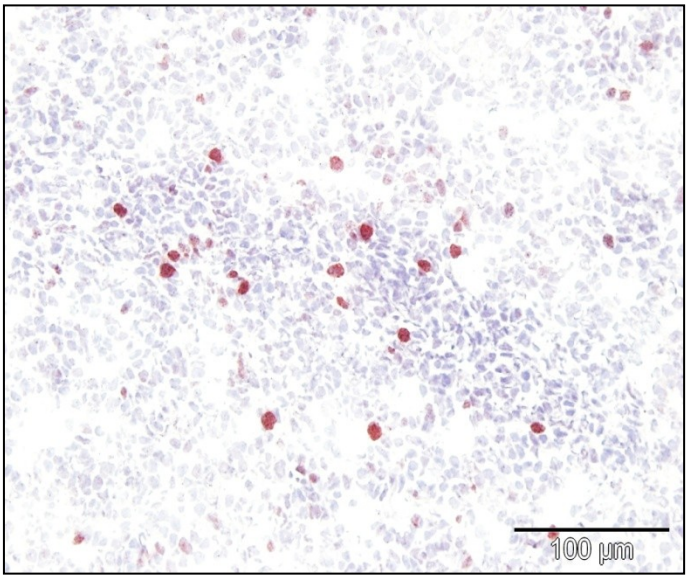

Fig. 8 c

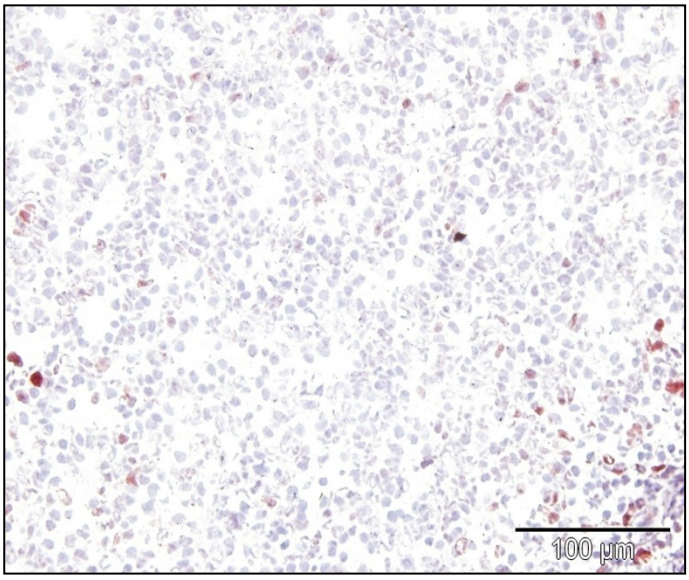

Fig. 8 d

Fig. 8 (c-d): Microscopic images of the **Ki67** immunohistochemical staining.

Upper image (Fig. 8c): **Placebo group**;

Lower image (Fig. 8d): **IGF-1 group**;

Magnification: 400-times; barr: 100µm

4. Discussion

The mortality and morbidity rate among infants that are born with CDH is still high due to the hypoplasia of the lungs and pulmonary hypertension. Although neonatal care has come a long way during the past years, a prenatal solution that promotes the growth and differentiation of the fetal lungs is sought after.

Since IGFs possess the ability of stimulating and regulating the development of fetal lung tissue, their prenatal application could depict a therapeutic approach for fetuses with CDH. The aim of our study was to evaluate this approach, using IGF-1 and -2, on the nitrofen model of the rat. However, the immunohistochemical protein expression of specific lung markers did not significantly differ between the treatment and control groups, thus leading to the assumption that intraamniotically applied IGFs have no beneficial effect on lung development and proliferation of CDH positive fetuses.

IGFs play a crucial role, not only as general growth factors during embryogenesis, but also in the process of maturation and development of the lungs, especially at a late fetal stage [28,29]. Studies with transgenic mice, that either lacked IGF-1, IGF-2 or one of their receptors, revealed different, but developed mostly lethal outcomes regarding pulmonary maturity. Sixty percent of the IGF-1 knockout mice died because of poor epithelial differentiation and reduced airway volume [30]. Mice carrying a null mutation of IGF-2 mostly suffered from alveolar malformations [31]. IGF-1R knockout mice were unable to survive after birth due to muscular hypoplasia resulting in an insufficient respiration [28,29]. Mice that lacked IGF-2R were prone to die of several organ- and lung abnormalities and were usually born 30% heavier than their wild-type relatives [32].

Nagata et al. took these results into consideration and aimed to investigate the effects of IGFs and its receptors on the lung development of fetal mice. The study was performed in vitro, where the lungs were cultured in a serum free-, an IGF-1 or an IGF-2 medium. By means of real time polymerase chain reaction (RT-PCR), the mRNA of the IGFs and their receptors were analyzed. Also, immunohistochemistry with the antibodies Ttf-1, SMA, anti-proliferating cell nuclear antigen (PCNA) and prosurfactant protein (proSp)-C, was carried out to examine the different serum groups. The highest levels of mRNA expression of IGF-1 and IGF-2 were found in the

early stages of lung development, while the mRNA of the receptors increased gradually and peaked at a later period of lung growth. The cultures containing the IGF-1 or IGF-2 serum both revealed better lung growth and differentiation. Ttf-1 and proSp-C were expressed in greater amounts in these two groups than in the serum free group, suggesting that the induced binding of the IGFs to their receptor might contribute to this reinforced expression. IGFs also stimulated the expression of SMA and PCNA, thus promoting angiogenesis and overall cell proliferation in the fetal lungs. Nagata et al. concluded that the IGFs contribute to a proper maturation of the lungs especially when binding to its receptors at an advanced period of lung development [26].

Esumi et al. went a step further and investigated the effect of IGFs on hypoplastic lungs of rats due to nitrofen induced CDH. After the rats were exposed to nitrofen, the fetuses were harvested and the lungs dissected and treated in vitro. They were divided into a serum free control group, an IGF-1 and an IGF-2 group. The mRNA expression of Ttf-1, SMA and T1α was measured with RT-PCR and the markers were also analyzed via immunohistochemistry. The results revealed a significantly higher expression of the mRNA of Ttf-1 and T1α in the IGF-2 group compared to the control group and the IGF-1 group. These markers relate to type 1 and 2 alveolar epithelial cells, thus indicating, that the administration of IGF-2 might contribute to an improved alveolar differentiation in CDH- impaired lungs [27].

In our study we tried to find out, if intraamniotically administered IGF-1, respectively IGF-2 would cause changes in protein expression of fetal lungs. Mainly based on the results of Esumi et al. and Nagata et al. we expected to obtain comparable changes. The protein markers used in our study were Ttf-1, Pdpn, SMA, Fgf-10 and Ki67. However, these markers did not show any significant differences in their expression patterns after the application of IGFs.

The reason, why intraamniotic treatment did not lead to changes in protein expression in our study could be caused by different factors.

The dosage (0,2 µg) of the applied IGF-1 and IGF-2 (on E19 und 20) might have been insufficient or the stimulus too short or too discontinuous to cause increasing proliferation of the fetal lungs.

Another reason could be a suppressive effect of the applied IGFs. Bloomfield et al. [33] reported that the intraamniotic application of IGF-1 caused a suppressive effect on circulating fetal IGF-1 levels. This effect also concerned IGF-1-mRNA levels in the liver, the muscle tissue and the placenta. The authors concluded, that *exogenic* IGF-1 could have suppressed *endogenic* IGF-1 levels. Perhaps this contributed to the missing effects of IGF in our study.

According to Kind et al. an infusion of IGF-1 during a treatment period of 10 days caused a decrease of mRNA expression of IGF-1 in the liver of fetal sheep. Moreover Bloomfield et al. showed that intraamniotically applied IGF-1 was taken up by the fetal gut into the blood circulation and also reached the fetal liver [34,12].

Possibly the expression of IGF-1 mRNA in the liver of rat fetuses could have caused a suppression of circulating IGF-1 levels in our study. Yakar et al. described the liver as the main source of IGF-1 in mice and this could also be possible in other rodents like rats [35]. Reduced IGF expression in liver could cause a reduction on systemic IGF levels.

Intraamniotically applied IGF could also have caused an effect on the placenta of the rats, since IGF receptors have been found there. After Sorem et. al. IGF causes stimulating effects on the transfer of proteins, the differentiation of trophoblast tissue and it leads to a reduced thromboxane production [36,37].

Skarsgard et al. described a suppressive effect of high levels of exogenously applied IGF-1 on endogenous IGF-1 levels and the sensitivity of the receptors. These receptors were downregulated during the application of IGF-1, which could also have influenced fetal growth [36,15].

Eremia et al. [14] investigated different ways of IGF-1 application and noticed that only intraamniotical application caused a significant increase of the fetal liver weight. Perhaps the way, dosage or the interval of application of IGF-1 and -2 caused suppressive effects on the fetal liver in our experimental study.

Maybe the administered IGF-1 and -2 was bound by different IGFBPs (perhaps mainly IGFBP-3) and could therefore not cause an increased protein expression in fetal lungs after two times of application. This speculation is based on an observation of Eremia et al., who described IGFBP-3 as the predominant binding protein in the

amniotic fluid and showed that production of IGFBP-3 is influenced by insulin, IGF-1 and nutrition. Increased concentrations of intraamniotic IGFBPs could have reduced the levels of the administered IGF and the uptake of IGF by the fetus in our study [14].

Bloomfield et al. described, that marked IGF-1 (125I-IGF-1) was taken up by fetal sheep and fastly distributed in the amniotic fluid. The amount of the IGF-1 bound to the IGFBPs was different among the animals, but even after 144 hours there was still a big part of the bound IGF-1 left. The most important binding protein was IGFBP-3. IGF-1 was swallowed by the fetus and taken up into the portal vein by fetal gut. 125I-IGF-1 was found in the amniotic fluid for 6 days in both, bound and free form. Bloomfield et al. concluded that 125I-IGF-1 was liberated continuously from the amniotic fluid [12].

The reason for the missing effects of IGF in our study could be the linkage to its IGFBPs or the degradation by different proteases.

Even a loss of the applied IGF through the placenta could be possible, because the amounts of 125I-IGF-1 in maternal plasma increased 12 hours after the injection [12].

Also, proteases in the amniotic fluid could be responsible for the degradation of the applied IGFs. Proteases like the serin protease split IGFBP-3, -4 and -5 and have been found in human amniotic fluid [38]. The levels of IGF could be influenced by IGFBPs, or vice versa the levels of IGFBPs by IGF-1. IGFBP-3 plays a crucial role in the regulation of the levels of IGF-1 in the amniotic fluid. According to Kimura et al. the bio-availability of 125I-IGF-1 summed up to about 9% of the applied dosage. The authors observed that the main part of IGF was taken into the portal vein by gut [38,39].

Nagata et al. reported on an increased proliferation of different cell types in the lungs of fetal rats. Elevated levels of SMA and TTF1 were found in the lung tissue, but these results were taken by cell culture in vitro and are not directly comparable with in vivo studies [26].

Harris et al. described the IGF-2R as an important clearance receptor for IGF-2. It binds IGF-2 and increases its lysosomal degradation [40]. Even an increased

degradation of IGF-1 through the IGF-2R cannot be ruled out. The IGF-2R binds IGF-1 with minor affinity, but could cause a certain clearance of IGF-1 [40,41].

However, the reasons for the missing effects of the applied IGFs in our study cannot be cleared without further analysis.

Further investigation would be important for finding out the influence of dosage, continuity and duration of the application of IGF-1 and -2. Measuring fetal and maternal blood levels of glucose and IGF-1 levels of the serum would probably contribute to analyzing the influence of the applied IGF on these parameters.

4.1. Conclusion

Summarizing the results of our study, intraamniotically applied IGF-1 and -2 did not lead to beneficial effects on lung proliferation and differentiation and is therefore not recommendable, at least in the performed protocol, as a treatment for fetal lung hypoplasia.

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