Retinoic Acid Attenuates the Mild Hyperoxic Lung Injury in Newborn Mice

Short title: Retinoic Acid Treatment of the Hyperoxic Lung Injury

M. Zimová, J.Mysliveček[1], P.Potměšil[2]

Neonatological Department of General Faculty Hospital, Prague, [1]Institute of Physiology, 1st Faculty of Medicine, Charles University, Prague, [2]Institute of Experimental Medicine, Department of Immunopharmacology, Academy of Sciences of the Czech Republic, Prague 4, Czech Republic

Corresponding author:
M.Zimová-Herknerová, Department of Neonatology,
Apolinářská 18, Praha 2,120 00.
E-mail: MagdalenaZ@seznam.cz

Summary

Newborn BALB/c mice were exposed to room air, 40% or 80% hyperoxia for 7 days. We tested retinoic acid (RA) as a potential factor capable to affect lung development and regeneration after hyperoxic injury and to maintain structural integrity of lung . RA functions in the control of gene transcription. The gene of vascular endothelial growth factor A (VEGF-A) is one of the RA responsive genes. Within three oxygen receiving groups one half received 500 mg/kg retinoic acid from day 3 to day 7 of the experiment and the others received placebo.We assessed the body weight (BW), the lung weight (LW) and the wet-to-dry lung weight ratio (W/D) on the seventh day. The expression of mRNA for the VEGF-A gene and the G3PDH, the housekeeping gene, was analysed by RT-PCR. Oxygen-exposed sham treated mice (80) weighted an average of 20% less than the room air exposed group at the end of the experiment - on the 7.day. However, the 80% hyperoxic group with the application of retinoic acid weighted only 13% less than the normoxic group. Both these differencies were statistically significant with P< 0. 0083 in comparison to control group and the two 80 and 80A groups differed between each other. (3. 98±0.15g =87% vs.3.66±0.26g=80% BW difference from the control group 4.66±0.16g=100%). The 80 and 80A W/D data did not differ between each other, although they both differed from control group and from 40 groups. (4.25±0.1; 4.33±0.2; vs.3.88±0.1). We found significant difference between 40 and 40A group and also the comparison to control group was different in 40 group and not in 40A group.(4.12±0.1 vs. 3.91±0.04). The 80A group had the expression of mRNA VEGF on the 64% of the control group. (145±26.2 OD vs. 229±48.4OD). The predictable decrease of the VEGF-A mRNA expression in the 80% hyperoxic sham treated group was to 41% of the control group. (94±11.7OD). There was no significant difference between 80 and 80A groups, although they both differed from all other groups. The normoxic and mild hyperoxic group RA-treated had the expression of one half higher than the normoxic sham treated group.We conclude that the retinoic acid treatment of newborn puppies of BALB/c mice exposed for 7 days to 80% hyperoxia reduced the growth retardation in the 80% hyperoxic group, reduced the W/D ratio in the 40% but not in the 80% hyperoxic group. The higher VEGF-A mRNA expression in the 80% hyperoxic group treated with RA was not of statistical significance.

Key words: retinoic acid, hyperoxia, vascular endothelial growth factor

Introduction

In our experiment, we exposed newborn mice to two degrees of hyperoxia (40% or 80%) and one half of them was treated with the retinoic acid. Neonatal response to hyperoxia is unique, probably because injury occurs during the period of alveolar development especially in mice. The newborn mouse is particularly well suited for studies of neonatal oxygen injury. Murine alveolar development begins on postnatal day 3, and saccular division is completed by the 14th postnatal day (Amy *et al*. 1977). This postnatal period, and the relative timing of alveolarization resembles human lung development (Warner *et al.* 1998).

The pathophysiology of lung injury during oxygen exposure is thought to include direct endothelial and epithelial cell damage by an increase in reactive oxygen species as well as release of products from activated leukocytes accumulating within the lung. Pathological effects of pulmonary oxygen toxicity include: atelectasis, edema, fibrin formation, inflammation, arterial thickening, bronchitis, alveolar cell hypertrophy.One important and uniquely neonatal response to hyperoxia is diminished alveolar development.

Experiments mainly in rodents showed that exogenous retinoic acid (RA) induces the formation of extra alveoli in newborn rats and maintains alveolar forming ability under inhibiting conditions like hyperoxia. (Massaro *et al.* 1996, Veness-Meehan *et al.* 2001). Alveolar regeneration with RA may be an important novel therapeutic appproach to the treatment of respiratory diseases characterized by a reduced gas-exchanging surface area such as bronchopulmonary dysplasia and emphysema for which there are currently no treatments.

Retinoids act through their specific nuclear receptors. RA functions in the control of gene transcription. The genes of VEGF family are RA responsive genes. The VEGF family of growth factors plays an essential role in both physiological and pathological angiogenesis. The archetypal and best characterized member of the VEGF family is VEGF-A.

VEGF-A is a mitogen for endothelial cells and is expressed by alveolar epithelial cells. VEGF-A plays an important role in normal lung development and adaptation. VEGF-A gene expression is regulated by oxygen levels. (Maniscalco *et al.* 1997) Chronic hypoxia has been shown to increase the expression of VEGF-A and its receptors VEGFR-1 and VEGFR-2 in the rat lung (Louzier *et al.* 2003). Since its original description, VEGF has been shown to have effects on a variety of non-endothelial cells, including alveolar epithelial cells. (Charnock-Jones 2005).

The links between RA and VEGF pathway are not yet completely understood. However, Massaro and his co-workers hypothesized that RA prevents the downregulation of VEGFR-2 (Clerch *et al*. 2004). The downregulation of VEGFR-2 is associated with a block in angiogenesis and an inhibition of alveolar septation.

The first aim of our study was to examine the effect of two degrees (40% or 80%) of hyperoxia on growth, lung injury and also the influence of two different degrees of hyperoxia on the VEGF-A mRNA expression in newborn mice. The second aim of our experiment was to compare these results between groups that received retinoic acid as a potential therapeutic agent and the other groups which received placebo.

Methods

Animals and Oxygen Exposure Protocol

The time dated pregnant BALB/c mice were maintained on standard laboratory food and water ad libitum. Lighting was set on 6:00 to 18:00 h, temperature at 28-30°C to achieve thermoneutral environment. Mice were kept in groups of one adult mother with 9-13 pups. 40% and 80% oxygen exposure was performed in the space of $1m^3$ Plexiglass chambers lined with lime soda into which oxygen was continuously delivered. The oxygen monitor (MiniOX ® 3000, MSA, USA) was set to tolerate 5% deviation of the desired oxygen concentration. The oxygen delivered to adult mothers and newborn pups was heated on temperature 37-39^{0}C and humidified at >70% relative humidity (humidifier Pegasus, UniNEB, USA). In normobaric hyperoxia, there is slightly less oxygen toxicity under conditions of humidified environment (>60% relative humidity) compared to dry gas oxygen exposure. Pulmonary oxygen toxicity, estimated by wet-to dry weight ratio , was less severe in a humid than in a dry atmosphere in normobaric oxygen. .(Lin et al., 1993). Exposure of rats to dry normobaric oxygen caused epithelial thickening in the lobar bronchi, which was not the case with humidified oxygen (Murchie et al., 1993).

Exposure to Hyperoxia.

Neonatal BALB/c mice were exposed to hyperoxia. The oxygen was obtained from Linde Technoplyn (Prague, Czech Republic). Within 12h of birth, pups from four to eight litters were pooled before being randomly redistributed to the newly delivered mothers.

The animals were divided into 6 groups: 1) room air without application of vitamin A (21) .2) room air with application of vitamin A (21A).3) 40% oxygen without application of vitamin A (40). 4) 40% oxygen with application of vitamin A (40A).5) 80% oxygen without application of vitamin A (80).6) 80% oxygen with application of vitamin A (80A). Nursing mothers were rotated between all groups every 24h to avoid oxygen toxicity in the mothers and to eliminate maternal effects between groups.

Application of vitamin A

Axetocal inj. ® (Biotika, retinoic acid) was administered intraperitoneally in the dose 500mg/kg once a day from day 3 to day 7 to the following three groups: 21A, 40A, 80A (Veness-Meehan *et al.* 2002). Vanees-Meehan treated newborn Sprague-Dawley rats with this dose of retinoic acid and found enhanced septal formation without evidence of effects on elastin gene expression after 4 week of recovery. This dose for a puppy of 4 g body weight presents 2mg=2940 IU dose intraperitoneally a day. The 500mg/kg dose=735k IU/kg is the highest dose used in the experiments with animal models of bronchopulmonary dysplasia which gives no adverse or toxic events of vitamin A and keeps the mice puppies alive. The vitamin adverse or toxic events include lethargy, vomiting, pallor, extremity tenderness, dry or scaly skin, or unusual pigmentation. We observed no adversed events of vitamin A treatment during the duration of the experiment.

The animals were weighed every day. The other groups of animals received placebo (cottonseed oil diluent) in the same amount.

At the seventh day of the experiment all animals were killed by decapitation. All protocols were approved by the institutional ethics committee.

Lung Wet Weight/Dry Weight Ratio (W/D)

The wet to dry lung ratio means in fact measurement of extravascular lung water. The lungs were excised, blotted dry, and weighed to obtain wet weight. The lungs were then dried in an oven at 60^0C and weighed daily. After 48 h, no further change in weight was observed in any sample.This weight was taken as dry weight for the calculation of the wet weight/ dry weight ratio. (Dhingra *et al.* 2001).

Histology of the lung

After the mice were killed, lungs were fixed in situ with 4% paraformaldehyde in phosphate-buffered saline (pH 7,4). Tracheas were cannulated and paraformaldehyde was instilled at 25cmH_2O pressure. After 5min, lungs were removed and further fixed overnight in 4% paraformaldehyde. The next day lungs were washed in PBS and serially dehydrated in increasing concentrations of ethanol before embedding in paraffin. Five micrometer tissue sections were stained with hematoxylin and eosin.

Reverse transcriptase – polymerase chain reaction (RT-PCR)

The expression of mRNA of vascular endothelial growth factor (VEGF) was assessed by semiquantitative reverse transcriptase polymerase chain reaction. Total RNA was isolated from approximately 100mg of lung tissue using the acid guanidinium thiocyanate-phenol-chloroform method (Chomczynski 1987). The yield and purity of RNA was quantified by measuring the ratio of the optical density at 260 and 280 nm. 0.5 μg of RNA was then reverse transcribed to complementary DNA (cDNA).

cDNA was synthetised using oligo 3´ primer of VEGF and recombinant Moloney murine leukemia virus reverse transcriptase in presence of RNAse inhibitor and then amplified by PCR.

Following reverse transcription, cDNA was amplified by the polymerase chain reaction with primers for VEGF and G3PDH, a housekeeping gene. Both reverse transcription and polymerase chain reaction were performed using the Thermocycler personal combi (Biometra, Germany). PCR reactions were performed with sequence specific oligonucleotide primers for mouse VEGF (forward primer 5´ ACA TCT TCA AGC CGT CCT GTG TGC 3´ , reverse primer 5´ -AAA TGG CGA ATC CAG TCC CAC GAG-3´). Primers for G3PDH, a reporter mRNA, were 5´-CCC ATC ACC ATC TTC CAG-3´ and 5´- ATG ACC TTG CCC ACA GCC-3´. The PCR reaction mix contained the forward and reverse primers (0.2 μM each), dNTPs (0.2 mM each), 10 mM Tris-HCl pH 8.8, 50 mM KCl, 1.5 mM $MgCl_2$, 1U of Taq DNA polymerase and 10 μl of cDNA in a total reaction volume of 50 μl. The reagents for the PCR reaction were obtained from Top-Bio, Prague, Czech Republic. The primers for VEGF-A and G3PDH were designed and produced by Biogen, Prague, Czech Republic. Thirty cycles were used for the PCR for VEGF gene as well as for the house keeping gene, G3PDH, according to the instruction of Biogen. The cycling temperatures and timings for VEGF gene were: 94 C for 60s, 62C for 90s, 72 C for 60s. The cycling temperatures and timings for G3PDH gene were: 94 C for 45s, 60 C for 45s, 72 C for 60s. After the last cycle, final extension at 72°C for 7 minutes was carried out.

The PCR products 563bp VEGF mRNA and 780bp G3PDH, a reporter mRNA, were identified by electrophoresis on 1.5 % agarose gel containing ethidium bromide. Only the samples with the positive expression of the housekeeping gene, G3PDH, were taken into account for further analysis. The gels were photographed in UV light and software BioDoc II digital (Biometra) was used for densitometric analysis of the bands. The expression of the G3PDH mRNA was constant through samples of all groups and the results of densitometric units of VEGF-A mRNA were counted as the VEGF-A/G3PDH ratio.

Statistical Analysis

The data are summarized as mean±SD throughout the text, tables and figures. Two way ANOVA with repetition was used to compare the six experimental groups. When multiple comparisons were made between means of two experimental groups , Bonferroni´s modification of t-test was applied to adequately assess the altered level of significance.

In Figure 6., the ratio of the expression of VEGF-A mRNA in the normoxic sham treated to the average G3PDH expression mRNA was defined as 100% (21 =control group) and all other groups are presented as a percentage of this group.

Results

Survival , growth, lung weight and W/D of neonatal mice exposed to normoxia, 40% and 80% hyperoxia treated or untreated with vitamin A.

All newborn mice survived 7 days in various degrees of hyperoxia, one adult mother mouse died on the sixth day of the experiment. The body weight of the pups from each group on the seventh day of the experiment is presented in Table 1. Neonatal exposure to 80% hyperoxia negatively affected growth. Oxygen-exposed sham treated mice (80) weighted an average of 20% less than the room air exposed group at the end of the experiment - on the 7.day. However, the 80% hyperoxic group with the application of retinoic acid weighted only 13% less than the normoxic group. Both these differencies were statistically significant by ANOVA analysis ($P<0,01$) and also the Bonferroni´s multiple comparison showed significant difference with $P< 0,0083$ in comparison to control group and the two 80 and 80A groups differed between each other. The mild hyperoxic groups (40% hyperoxia) regardless of vitamin A application did not differ in body weights compared to the normoxic groups and both differed to 80 groups in Boferroni´s multiple comparison.

As shown in Table 1., after 7days of the experiment the lung wet weight was highest in the 80% hyperoxic sham treated group. The ANOVA analysis (Table 2) describes high statistical significance of both factors (RA treatment and hyperoxia) and its combination, and we did not make any more statistics of lung wet weight.

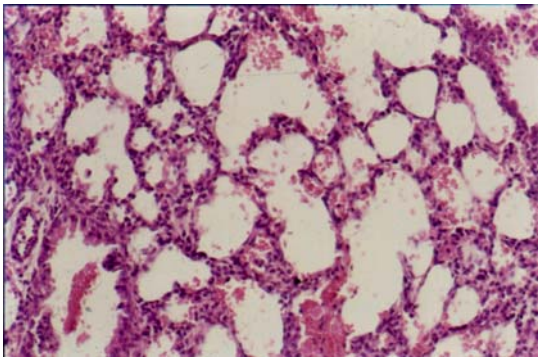
The W/D ratio according to ANOVA is not different between RA and sham treated groups. However, when Bonferroniś multiple comparison was made we found difference between 40 and 40A group and also the comparison to control group was different in 40 group and not in 40A group. The 80 and 80A data did not differ between each other, although they both differed from control group and from 40 groups.

Histology of the lung

Both normoxic group 21 and 21As (Figure 1.):

Typical structure of neonatal mouse lung tissue. No diference between these two groups.
40A: Typical structure of neonatal mouse lung with narrow alveolar walls and interalveolar septa and adequate secondary septation.

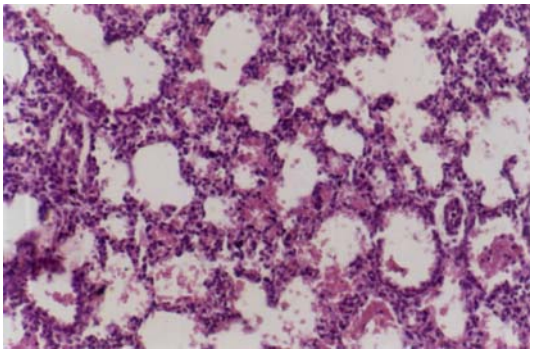
Figure 1.:



The mild hyperoxic sham-treated group- 40 (Figure 2.):

Lung tissue with thickened alveolar walls and interalveolar septa. No alveolár simplification, adequate secondary septation. Some degree of pulmonary interstitial edema.

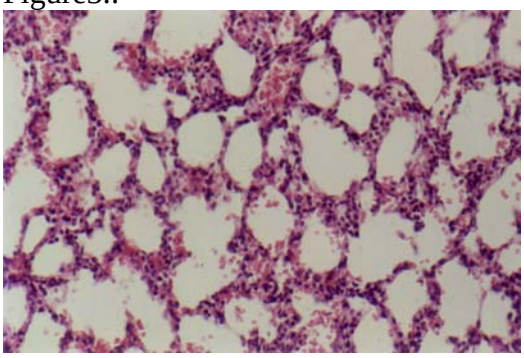
Figure 2.:



The 80% hyperoxic sham- treated group-80 (Figure 3.):

Lung tissue with enlarged air spaces, thickened alveolar walls and a few secondary crests. Inflammatory cells present in alveoli. Interstitial pulmonary edema. Hyaline membranes present focally. Squamous epithel metaplasia in bronchi.

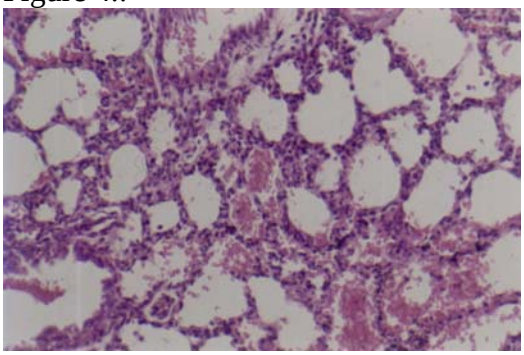
Figure3.:



The 80% hyperoxic vitamin A treated group-80A (Figure 4.):

Lung tissue with narrow alveolar walls and septa, adequate formation of secondary crests.Inflammatory cells present in alveoli. Interstitial pulmonary edema.

Figure 4.:



Analysis of the mRNA VEGF expression

The densitometrical analysis of the RT PCR bands assessed by the software BioDoc II digital (Biometra) is presented in Table 1. The normoxic and mild hyperoxic RA-treated groups had the expression of one half higher than the normoxic sham treated group. There was no decrease of the mRNA VEGF-A in the 40% hyperoxic sham-treated group at the end of our experiment. The 80A group had the expression of mRNA VEGF on the 64% of the control group. The predictable decrease of the VEGF-A mRNA expression in the 80% hyperoxic sham treated group was to 41% of the control group.

ANOVA analysis supports the important role of vitamin A in the VEGF-A gene regulation. However, in the range of multiple comparison of these data there was no significant difference between 80 and 80A groups, although they both differed from all other groups. On the other hand, we can say that VEGF-A gene is a vitamin A responsive gene.We found significant difference of 40A and 21A groups from 40 and 21 groups.

Figure 5.: Expression of mRNA VEGF and G3PDH, a housekeeping gene, in different groups.
OD-optical density. Blue columns=G3PDH. Lila columns=VEGF-A.

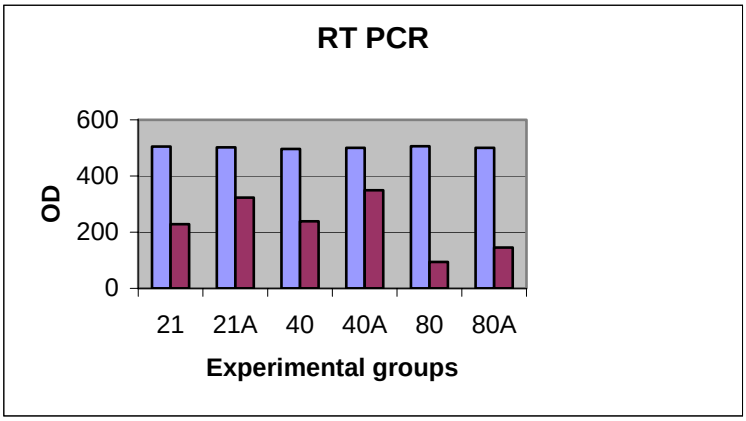
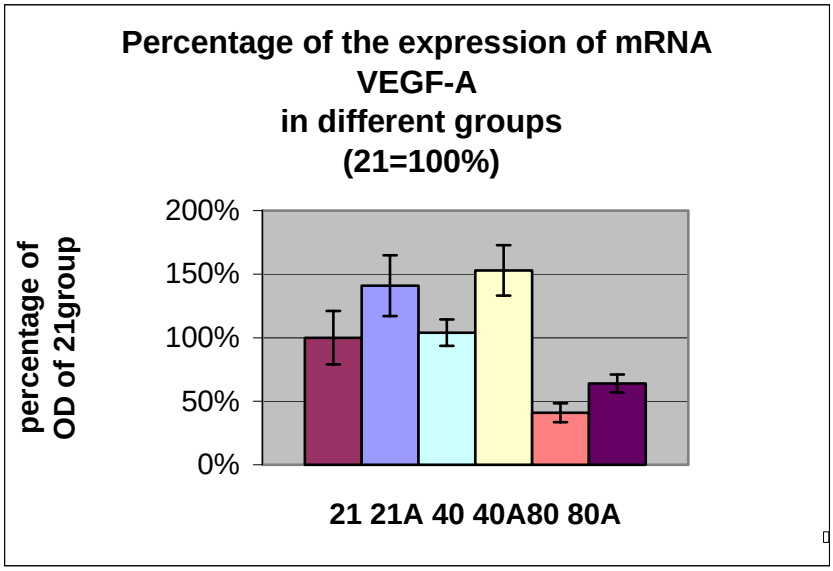


Figure 6: Percentage of the expression of mRNA VEGF-A in different groups (21=100%)



Discussion

In this experiment, we tested whether retinoic acid could act as an useful agent in preventing the hyperoxic injury of the developing lung. We exposed the animals to 40% or 80% normobaric hyperoxia for 7 days, in order to describe the difference between these two hyperoxic injuries as well as the difference between the action of RA in mild or high hyperoxia. The expression of the mRNA VEGF-A was measured by RT-PCR to identify whether retinoic acid can protect the developing lung from the hyperoxic injury by preserving the expression VEGF-A gene.

The mechanism by which RA prevents hyperoxia induced growth inhibition probably interferes with TGFbeta signalling pathway. As described in a number of reports oxidative stress is associated with the induction of activation of the TGFbeta pathway. TGFbeta actions are well defined by their ability to inhibite epithelial cell growth in various tissues including lungs (Chinoy M 2003). Recent papers suggest that RA antagonizes TGFbeta pathway by reducing TGFbeta receptor expression in hyperoxia exposed cells. 40% hyperoxia did not affect growth regardless of RA application. We speculate that 40% hyperoxia is not strong enough to activate TGF beta signalling pathway.

The 80% hyperoxic adverse effect on growth of the animals presented in this paper is thus in accord with other recent papers on this topic. Warner reported about an average 8% body weight difference of newborn mice exposed to 85% hyperoxia for one week. Our results regarding the body weight are stronger- we found 20% body weight loss compared to the control group in the 80% hyperoxic group and 13% in the 80% hyperoxic group treated with RA. Our results regarding the body weight in the 80A group are closer to Warnerś results. There were only 60% surviving animals after the first week in Warnerś experiment. Warner´s experiment was done with different tribe (FVB/A) of mice. The BALB/c mice we used in this experiment might be less sensitivitive to the hyperoxic lung injury.

The lung weight enlargement during hyperoxia is attributed principal to an extravascular, interstitial accumulation of water, sodium and plasma proteins since the cellular mass of pulmonary parenchyma did not increase significantly.(Clark and Lambertsen 1971). Our results of LW after 7 days of the experiment confirmed lung volume enlargement in all hyperoxic groups. We found statistically significant difference between LW in RA treated and sham-treated groups by ANOVA analysis. Our histological findings are also in accord with these principles, we found pulmonary interstitial edema in all hyperoxic groups, however not in the 40A group.

The lung weight was measured for example by Veness-Meehan (Veness-Meehan *et al.* 2002)in her experiment. However, she presents results after 4 weeks recovery phase from hyperoxia. She reported the 50% enlargement of LW in the hyperoxic group and little enlargement of LW in the hyperoxic group treated with RA-not statistically significant difference compared to the control group. We can speculate about the importance of our presented difference between LW in RA treated and sham-treated groups measured just after the exposure to hyperoxia. This result might reflect some degree of therapeutic effect of RA already during the exposure of hyperoxia.

Our results found the presumable increase of W/D in the 80% hyperoxic group. This increase was detected regardless of the application of vitamin A. Vitamin A did not prevent the edematous lung injury in the exposure to 80% hyperoxia for one week. 40% hyperoxia is also responsible for some degree of lung injury according to our results of lung histology and the W/D ratio of the lungs. These results evoke the possibility that retinoic acid might have protective anti-edematous effects in 40% but not in 80% hyperoxic conditions in the presented design of hyperoxic model.

The VEGF-A mRNA expression was statistically different in both 80% hyperoxic groups compared to normoxic group. The 80A group amount of VEGF-A mRNA transcripts was 20% higher than in 80 group. However, this difference was not of statistical significance. We have no support that retinoic acid prevents the decrease in VEGF-A gene expression in the 80 hyperoxic condition to some level. On the other hand, our results in 40A and 21A group show that VEGF-A is a RA responsive gene. These results are in accord with other recent papers on this topic. Greater capillary surface density and VEGF-A gene expression

was observed in lungs of preterm lambs receiving retinoic acid under hyperoxic condition than in lungs of untreated lambs. (Bland *et al.* 2000, 2003, 2003).

Exogenous provision of RA to explants in whole mouse embryo culture restored vascular remodeling, as well as the signalling pathways that control endothelial growth. Exogenous provision of VEGF-A failed to rescue endothelial cell proliferative control but collectivelly promoted vascular remodelling, suggesting that these processess are regulated via a signalling hierarchy downstream of RA (Bohnsack *et al.* 2004).

We asked if our results of lung histology, wet weight and W/D state any information about the preserved endothelial cell function. In the 80% hyperoxic group our LW, W/D and RT PCR VEGF-A results do not evoke any general conclusions because the difference between RA treated and sham treated groups in the RT PCR VEGF-A parameter is not of statistical significance according to Bonferroni´s comparison. We intend to do further experiments with a longer interval of 80% hyperoxia exposure, for example two weeks and give these parameters together.

Conclusion

We conclude that the treatment of newborn puppies of BALB/c mice exposed for 7 days to 40% or 80% hyperoxia with retinoic acid from 3. to 7. day of life reduced the growth retardation in the 80% hyperoxic group, reduced the W/D ratio in the 40% but not in the 80% hyperoxic group and reduced to some level the decline in the VEGF-A mRNA expression in the 80% hyperoxic group compared with the untreated group.

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References

AMY RW, BOWES D, BURRI PH, HAINES J, THORLBECK WM: Postnatal growth of the mouse lung. *J*: *Anat* **124**: 131-151, 1977.

BHATT AJ, PRYHUBER GS, HUYCK H, WATKINS RH, METLAY LA and MANISCALCO WM: Disrupted Pulmonary Vasculature and Decreased Vascular Endotelial Growth Factor, Flt-1, and TIE-2 in Human Infants Dying with Bronchopulmonary Dysplasia.
Am J Reaspir Crit Care Med **164**: 1971-1980, 2001.

BLAND RD, ALBERTINE KH, PIERCE RA, STARCHER BC, CARLTON DP: Impaired Alveolar Development and Abnormal Lung Elastin in Preterm Lambs with Chronic Lung Injury: Potential Benefits of Retinol Treatment. *Biol Neonate* **84:** 28-29, 2003.
BLAND RD, ALBERTINE KH, CARLTON DP, KULLAMA L, DAVIS P, CHO SC, KIM BI, DAHL M, TABATABAEI A: Chronic Lung Injury in Preterm Lambs : Abnormalities of the Pulmonary Circulation and Lung Fluid Balance. *Pediatr Res* **48**: 64-74, 2000.

BLAND RD, LING CY, ALBERTINE KH, CARLTON DP, MAC RITCHIE AN, DAY RW, DAHL M: Pulmonary Vascular Dysfunction in Preterm Lambs with Chronic Lung Disease.
Am J Physiol Lung Cell Mol Physiol **29**: 312-318, 2003.
BOHNSACK BL, LAI L, DOLLE P, HIRSCHI KK: Signaling Hierarchy Downstream of Retinoic Acid that Independently Regulates Vascular Remodeling and Endothelial Cell Proliferation. *Genes and Development* **18**: 1345-1358, 2004.
CHINOY MR: Lung Growth and Development. *Front in Bioscience* **8**: 392-415, 2003.
CHINOY MR, TUDHOPE S, BHABUTA R, CILLEY RE, CHENG ZH, MILLER SA, MYERS RL: Role of Retinoic Acid and Its Receptors in Murine Lung Morphogenesis. *Am J Respir Crit Care Med* **161**: A564, 2000a.
CHINOY MR, TUDHOPE S, CHENG ZH, MYERS RL: Influence of Retinoic Acid in Human Fetal Differentiation. *Am J Respir Crit Care Med* **161**: A563, 2000b.
CHINOY MR, ZGLESZEWSKI SE, CILLEY RE, KRUMMEL (tm): Dexamethasone Enhances Ras-Recision Gene Expression in Cultured Murine Fetal Lungs: Role in Development. *Am J Physiol Lung Cell Mol Physiol* **279**: L312-L318, 2000.
CHARNOCK-JONES SD: Vascular Endothelial Growth Factors (VEGFs) Their Receptors and Their Inhibition. *Celltransmissions* **21/1**: 1-7, 2005.
CHOMCZYNSKI P, SACCHI N: Single step method of RNA isolation by acid guanidium thiocyanate-phenol-chlorophorm extraction. *Anal Biochem* **162**: 156-159, 1987.
CLARK JM, LAMBERTSEN CJ: Pulmonary Oxygen Toxicity: A Review. *Pharmacological Reviews* **23/2**: 43-48, 1971.
CLERCH LB, BARAS AS, MASSARO GD, HOFFMAN EP, MASSARO D: DNA Microarray analysis of Neonatal Mouse Lung Connects Regulation of KDR with Dexamethasone-Induced Inhibition of Alveolar Formation. *Am J Physiol Lung Cell Mol Physiol* **286(2)**: L 411-L419, 2004.
DHINGRA VK, UUSARO A, HOLMES CL, WALLEY KR: Attenuation of Lung Inflammation by Adrenergic Agonists in Murine Acute Lung Injury. *Anesthesiology* **95**: 947-953, 2001.
LE CRAS TD, MARKHAM NE, TUDER RM, VOELKEL NF, ABMAN SH: Treatment of Newborn Rats with a VEGF Receptor Inhibitor Causes pulmonary Hypertension and Abnormal Lung Structure. *Am J Physiol Lung Cell Mol Physiol* **283**: L 555-L 562, 2002.
LIN Y, JAMIESON D: Effect of humidity on hyperoxic toxicity. *J Appl Physiol* **75:** 1980-1983, 1993.
LOUZIER V, RAFFESTIN B, LEROUX A, BRANELLEC D, CAILLAUD JM, LEVAME M, EDDAHIBI S, ADNOT S: Role of VEGF-B in the Lung during Development of Chronic Hypoxic Pulmonary Hypertension. *Am J Physiol Lung Cell Mol Physiol* **284**: L926-L937, 2003.
MANISCALCO WM, WATKINS RH, DANGIO CT, RYAN RM: Hyperoxic injury decreases alveolar epitelial cell expression of vascular endotelial growth factor (VEGF) in neonatal rabbit lung. *Am J Respir Cell Mol Biol* **16**: 557-567, 1997.
MASSARO D, MASSARO DE CARLO G: Retinoids, Alveolus Formation, and Alveolar Defficiency-Clinical Implications. *Am J Respir Cell Mol Biol* **28**: 271-274, 2003.
MURCHIE P, JOHNSTON PW, ROSS JAS AND GODDEN DJ: Effects of hyperoxia on bronchial wall dimensions and lung mechanics in rats. *Acta Physiol Scand* **148**: 363-370, 1993.
NABEYRAT E, CORROYER S, BESNARD V, CAZALS-LAVILLE V, BOURBON J, CLEMENT A: Retinoic Acid Protects against Hyperoxia Mediated Cell-Cycle Arrest of Lung Alveolar Epithelial Cells by Preserving Late G1 Cyclin Activities. *Am J Respir Cell Moll Biol* **25**: 507-514, 2001.

PARKER TA, ABMAN SH: The Pulmonary Circulation in Bronchopulmonary Dysplasia. *Seminars in Neonatology* **8**: 51-62, 2003.
VENESS-MEEHAN KA, PIERCE RA,MOATS-STAATS BM, STILES AD: Retinoic Acid Attenuates O_2 – Induced Inhibition of Lung Septation. *Am J Physiol Lung Cell Mol Physiol* **283**: L971-980, 2002.
WARNER BB, STUART LA, PAPES RA, WISPE JR: Functional and pathological effects of prolonged hyperoxia in neonatal mice. *Am J Physiol* **275** (*Lung Cell Mol Physiol* **19**): L110-L117, 1998.
ZVAROVA J: Základy statistiky pro biomedicínské obory. *Karolinum.* Prague, 1998.

Table 1. : Body weight, lung wet weight, wet/dry lung ratio, OD mRNA of VEGF-A assessed by RT-PCR and the VEGF-A/G3PDH ratio of the 7 days old mice of each group:

Group	21	21A	40	40A	80	80A
Number	10	10	10	10	10	10
BW(g)seventh day	4.65±0.15	4.57±0.2	4.7±0.18	4.6±0.21	3.67±0.26*	3.98±0.15
LW(mg)	61.72±1.8	63.71±2.6	70.3±2.7	65.6±1.78	93.6±1.45	79.9±0.8
W/D	3.88±0.09	3.93±0.11	4.12±0.1*	3.91±0.04	4.6±0.09	4.37±0.2
VEGF-A mRNA (OD)	244±58.4	319±40	238±24*	349±32.4	94±12.4	138±22.7
VEGF-A/G3PDH	0.48	0.64	0.47	0.7	0.2	0.3

- weight,LW=lung weight,W/D data are summarized as mean±SD
- BW=body =lung wet to dry weight ratio
- *p<0.0083 in Bonferroniś comparison with control group (=21) and respective oxygen level RA treated group

Table2: Two way ANOVA with repetition

Factor	Vitamin A treat placebo	Concentration of inhaled oxygen	Interaction of two factors
BW	0.31	8.23E-19	0.007
LW	3.38E-14	5.33E-19	5.37E-16
W/D	0.602	5.16E-16	5.09E-05
VEGF-AmRNA	5.42E-11	1.46E-22	0.018

Presented data are p levels of the two way ANOVA analysis with repetition