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Evolution of Gene Expression Changes in Newborn Rats after Mechanical Ventilation with Reversible Intubation

THESE

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Evolution of Gene Expression Changes in Newborn Rats after Mechanical Ventilation with Reversible Intubation

Synthèse

Résumant mon travail de thèse, l'article qui suit décrit un nouveau modèle animal servant à étudier l'impact combiné d'une ventilation mécanique (VM), d'une oxygénothérapie et d'une inflammation sur des poumons immatures. Cette étude permet, pour la première fois, de mesurer l'expression de gènes à distance d'une VM pour en analyser la cinétique.

La VM représente un traitement intégral dans la prise en charge de prématurés. Sauvant des vies, elle est cependant non-physiologique et décrite comme nocive à court et à long terme, empêchant le bon développement pulmonaire. Nombreuses études se sont intéressées à l'impact immédiat de la VM sur les poumons, mais il n'existe à ce jour aucun modèle de rongeur pour en analyser les effets tardifs.
Par analogie avec la clinique, nous avons créé un modèle avec un animal dont le stade développemental pulmonaire est comparable aux prématurés humains et consistant en une oxygénothérapie, une VM modérée avec intubation non chirurgicale, similaire à la pratique quotidienne, et un contexte inflammatoire mimant celui de chorioamnionite dans lequel bien des prématurés naissent. Nous avons ensuite réalisé une extubation pour permettre une période de rétablissement, puis fait des analyses et sur le plan structurel par histologie conventionnelle et en 3D, et sur le plan biologique, par analyse de l'expression de gènes et de protéines.

Ce travail a permis de valider ce nouveau modèle comme outil de recherche pour réaliser des mesures à distance d'une VM chez des rats nouveau-nés. Comparant ces mesures à celles prises à la fin de la VM, nous observons: une augmentation initiale et transitoire des médiateurs impliqués dans la cascade inflammatoire dont le corrélat histologique est une maladie inflammatoire pulmonaire et, tardivement, une altération plus développementale de la structure pulmonaire avec diminution de l'alvéolarisation. Ceci pourrait être en partie dû à une expression asynchrone de gènes décrits comme importants pour la formation des alvéoles (matrix metalloproteinase 9, elastine).

Offrant une nouvelle approche pour la recherche pulmonaire chez les rongeurs, ce modèle servira comme futur outil pour approfondir nos connaissances de la physiopathologie conduisant aux altérations structurelles retrouvées dans les poumons d'anciens prématurés soumis à une VM (dysplasie broncho-pulmonaire), pour tester l'influence de certains traitements (p.ex. surfactant) et pour étudier les effets de la VM en l'appliquant à des modèles transgéniques.

Evolution of Gene Expression Changes in Newborn Rats after Mechanical Ventilation with Reversible Intubation

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Key words:
Lung development
Ventilator-Induced Lung Injury
LPS
Chronic Lung Disease

Running head: Evolution of gene expression after mechanical ventilation

Abstract

Mechanical ventilation (MV) is life-saving but potentially harmful for lungs of premature infants. So far, animal models dealt with the acute impact of MV on immature lungs, but less with its delayed effects. We used a newborn rodent model including non-surgical and therefore reversible intubation with moderate ventilation and hypothesized that there might be distinct gene expression patterns after a ventilation-free recovery period compared to acute effects directly after MV. Newborn rat pups were subjected to 8h of MV with 60% oxygen (O_2), 24h after injection of lipopolysaccharide (LPS), intended to create a low inflammatory background as often recognized in preterm infants. Animals were separated in controls (CTRL), LPS-injection (LPS) or full intervention with LPS and MV with 60% O_2 (LPS+MV+O_2). Lungs were recovered either directly following (T:0h) or 48h after MV (T:48h). Histologically, signs of ventilator-induced lung injury (VILI) were observed in LPS+MV+O_2 lungs at T:0h, while changes appeared similar to those known from patients with chronic lung disease (CLD) with fewer albeit larger gas exchange units, at T:48h. At T:0h, LPS+MV+O_2 increased gene expression of pro-inflammatory MIP-2. In parallel anti-inflammatory IL-1Ra gene expression was increased in LPS and LPS+MV+O_2 groups. At T:48h, pro- and anti-inflammatory genes had returned to their basal expression. MMP-2 gene expression was decreased in LPS and LPS+MV+O_2 groups at T:0h, but no longer at T:48h. MMP-9 gene expression levels were unchanged directly after MV. However, at T:48h, gene and protein expression increased in LPS+MV+O_2 group.. In conclusion, this study demonstrates the feasibility of delayed outcome measurements after a ventilation-free period in newborn rats and may help to further understand the time-course of molecular changes following MV. The differences obtained from the two time points could be interpreted as an initial transitory increase of inflammation and a delayed impact of the intervention on structure related genes.

Introduction

Respiratory insufficiency in preterm newborns mainly arises from an insufficient gas exchange surface and an immature air-blood barrier aside from the effects of immature respiratory drive and insufficient surfactant production. Indeed, human alveolar formation starts only after about 36 weeks of gestation.[1] Furthermore, prematurity is often associated with some degree of inflammation which has a potential impact on their postnatal respiratory abilities.[2,3] Although life-saving, mechanical ventilation (MV) in preterm patients corresponds to the non-physiologic application of positive pressure to immature lungs in the saccular stage. This may provoke, in the acute phase, enhanced inflammation and epithelial lesions known as ventilator-induced lung injury (VILI) but may also have an impact on lung development with long-term sequelae. The resulting chronic lung disease (CLD), or "new" bronchopulmonary dysplasia (BPD), is characterized functionally by prolonged oxygen dependency and histologically by decreased alveolarization with a lower number and an augmented size of alveoli, and a disrupted angiogenesis.[4]

During the last two decades, authentic animal models of preterm lambs and baboons have been developed to investigate the effects of MV and oxygen on lung development. They confirmed the essential features of CLD and their association with MV and oxygen use.[5-8] To varying degrees, the ventilated lungs also manifested signs of VILI, with structural changes and increased concentration of pro-inflammatory cytokines in tracheal aspirates and homogenized lung tissue.[9,10] Among the cytokines considered, Interleukin-8 (IL-8) or its rodent counterpart MIP-2 (CXCL2), the most potent leukocyte chemoattractant and also a regulator of angiogenesis, was increased in almost all models of MV. Blockade of its receptor CXCR2 attenuated VILI after MV.[11] IL-6, which has both pro- and anti- inflammatory properties, was predictive of ARDS severity.[11] IL-1 still has a debated role, but seemed to worsen VILI. IL-10 finally had a protective effect in multiple models of

acute lung injury.[11,12] On a micro-structural level, accumulation of disorganized elastin protein was reported, due to changes in tropoelastin gene expression and an increased elastase activity.[13]

Rodent models also became a cornerstone for investigating the effects of MV and oxygen on lung tissue, for various reasons, including the well described analogy in lung developmental stages of rats/mice compared to human, as well as economical advantages and the capacity of fast reproduction. Lungs of newborn rats and mice are in the saccular stage but produce already sufficient endogenous surfactant. Different rodent models were used to study the influence of MV on the alveolarization process, and considered similar to the situation of ventilated preterm infants after exogenous surfactant treatment. These rat studies added important and more comprehensive knowledge about pathways and mechanisms involved in lung development as well as potential treatment approaches focusing on inflammation, vascular development, structural components and cell death.[14-19]

The use of newborn rodent models with increased time of MV ranging from 8 to 24 hours allowed the investigation of developmental aspects of MV. Recent studies confirmed an arrest of alveolar formation and a dysfunction in angiogenesis due to prolonged MV with or without oxygen.[20,21] Kroon et al. identified cell cycle arrest as a potential mechanism of disturbed lung development and Hilgendorff and colleagues highlighted the importance of the extracellular matrix (ECM) and in particular the elastin metabolism in the pathogenesis of VILI and CLD.[22,23]

However, common to all these newborn rodent models is the fact that outcome was measured immediately after MV and none of them so far addressed the question of delayed effects of MV on lung development after a ventilation-free recovery period. To fill this gap and to come as close as possible to the clinical reality in neonatal care of preterm infants, we tested a newborn rat ventilation model with a reversible, non-surgical intubation procedure with moderate MV. Twenty-four hours after an injection

of lipopolysaccharides (LPS), intended to create a low inflammatory background similar to the inflammatory context encountered by a high rate of preterm babies at birth, newborn rats were ventilated for 8 hours. Then, they were weaned off of anesthesia and MV and extubated to spontaneous respiration. Outcome measurements were performed two days later. We hypothesized that with this delayed analysis, specific time dependent gene expression changes might be identified. Here we present the first results of this model showing a distinct expression pattern of inflammatory and ECM genes directly at the end and with a 48h delay following the MV intervention.

Material and Methods

Animal intervention procedures: Pregnant female Wistar rats (Charles River Laboratories, L'Arbresle Cedex, France) were housed in the animal facility in a controlled 12h light-dark cycle with food and water supplied ad libitum. To ensure comparable weight gain, litter size was reduced and kept constant to 8-10 pups per litter. Pups were marked on P3 and randomly assigned to the intervention group (LPS+MV+O_2), the LPS group (LPS injection, but no MV) or the control group (CTRL). Four litters were dedicated to each time point, with 28 animals included at T:0h (9 Ctrls, 9 LPS+VM+O_2, 10 LPS group) and 22 at T:48h (8 Ctrls, 6 LPS+VM+O_2, 8 LPS group). Twenty-four hours before initiation of MV, animals of the intervention and the LPS group received an intraperitoneal injection of 3mg/kg LPS (E. coli serotype 026:B6; Sigma-Aldrich Inc., St.Louis, USA) while controls were injected the same volume of NaCl 0.9%. The day of ventilation (P6 or P7), the pups of the intervention group were anesthetized with a mixture of 50 mg/kg ketamine (Ketaminol® 10, Intervet, Swine, Skovlunde, Denmark), 1 mg/kg acepromazine (Prequillan®, Arovet AG, Zollikon, Switzerland) and 0.08 mg/kg atropine (Hänseler AG, Herisau, Switzerland). An orotracheal intubation was performed under direct laryngoscopy with a 24G i.v. catheter (Optiva® W I.V.Catheter, Smiths Medical International Ltd. Rossendale, UK). The tube was then connected to a ventilator (Voltek Rodent Ventilator, Voltek Enterprises Inc, Toronto, Canada) in volume-controlled mode with a tidal volume of 10 ml/kg.[24] Respirator settings are shown in Table 1. Oxygen concentration (60%) was verified in the inspiration circuit at the beginning of each experiment by an external oxymeter (Mono 2, Roche Diagnostics, Basel, Switzerland). Anesthesia was maintained by adding 1–2% of Isoflurane® (Baxter, Volketswil, Switzerland) to the inspiration circuit using a Combi-vet® evaporator (Rothacher and Partner Electronics, Bern, Switzerland). During the eight hours of MV, heart rate (Hewlett-Packard GmbH, Dübendorf, Switzerland) and rectal

temperature (Dynatime SA, La Chaux-de-Fonds, Switzerland) were continuously monitored. Body temperature was maintained at 34-36°C by thermal blanket and heating lamp. To prevent dehydration, the animals were covered by a small incubator and fluid was supplied every 2.5 hour by ip injection of 5-10 ml/kg body weight Ringer's lactate (B.Braun Medical AG, Sempach, Switzerland). Animals of the control and the LPS-group were also separated during eight hours from mother and siblings in a warm environment and had an equivalent number of ip injections of Ringer's lactate. Animals assigned to the group of delayed outcome assessment (T:48h) were weaned off stepwise over 30 minutes of Isoflurane® at the end of the ventilation period. When efficient spontaneous breathing had reappeared, extubation was performed. As soon as general behavior had become normal, the pups were returned to mother and siblings. Physiologic feeding habits were resumed soon after putting the pups back with the mother. Starting at P3 and throughout the whole experimental protocol, the rat pups underwent a daily clinical examination and measurement of body weight.

Table 1: Ventilation parameters

Ventilation parameters	
Respiratory rate (RR) (per min)	60
Ratio of Inspiratory to Expiratory time (I:E)	1:1
Fractional inspired Oxygen (FiO_2)	0.6
Tidal volume (Vt) (ml/kg)	10
Positive End Expiratory Pressure (PEEP) (cm H_2O)	3.9 ± 0.7
Peak Inspiratory Pressure (PIP) (cm H_2O)	26.8 ± 3.6
Mean Airway Pressure (MAP) (cm H_2O)	10.9 ± 1.5

Values are expressed as mean ± standard deviation from ≥6 independent animals.

Institutional review: All animal procedures were conducted according to the "Swiss Legislation for Animal Protection Act". The detailed protocol was approved by the responsible Veterinary Commission of the Canton of Vaud.

Preparation of lung tissue: The excision and preparation of lung tissue was done as described previously.[16] In brief, following an overdose of pentobarbital, an endotracheal tube was inserted by tracheotomy, the thorax opened and the left main bronchus ligated. The left lung was excised, cut in two pieces for gene and protein analysis, snap frozen in liquid nitrogen and stored at -80°C. The remaining right lung was infused with 4% paraformaldehyde (PFA) in PBS through the tracheally introduced 24G catheter by maintaining a constant pressure of 20 cmH_2O in the lung to equally fill the entire right lung. Under constant pressure conditions, the tube was then removed and the trachea immediately ligated to maintain pulmonary lobes at a constant volume. The excised right lung was immersed in 4% PFA. The fixed lung was separated into individual lobes for histology.

RNA extraction and quantitative RT-PCR: Total lung RNA was extracted with TRIzol (Invitrogen Corp, Carlsbad, California), following the standard protocol. With Prime Script 1st strand cDNA synthesis kit (Takara BIO Inc, Otsu, Shiga, Japan) 1 µg RNA was reverse-transcribed. 50 ng cDNA was amplified using the Absolute qPCR SYBR Green mix (ABgene Ltd, Epsom, United Kingdom) in a Rotor-Gene 600 PCR device. Analysis of the results was made with the Rotor-Gene 600 Series Software 1.7, using the standard curve method (Qiagen GmbH, Hilden, Germany). Primer sequences, identified with Primer-BLAST software (National Center for Biotechnology Information; www.ncbi.nlm.nih.gov), are summarized in Table 2.

Table 2: Primer Sequences

Gene	Accession Number	Forward Primer Sequence	Reverse Primer Sequence
GAPDH	NM_017008.3	AGACAGCCGCATCTTCTTGT	CTTGCCGTGGGTAGAGTCAT
MIP-2	NM_053647.1	CCAACCATCAGGGTACAGGG	GGGTCGTCAGGCATTGACA
IL-1β	NM_031512.2	CTTGTCGAGAATGGGCAGTCT	TGTGCCACGGTTTTCTTATGG
IL-6	NM_012589.1	CCGGAGAGGAGACTTCACAG	CAGAATTGCCATTGCACAAC
IL-1Ra	NM_022194.2	CAAGCGCTTTACCTTCATCC	GGCTCTTTTGGTGTGTTGGT
IL-10	NM_012854.2	CCGAGAGCTGAGGGCTGCCT	TGGTTCTCTGCCTGGGGCATCA
TNF-α	NM_012675.3	CCTCTTCTCATTCCTGCTCG	CCCATTTGGGAACTTCTCCT
Tropo-elastin	NM_012722.1	GCTGATCCTCTTGCTCAACC	CTGGCCTTGAAGCATAGGAG
MMP-2	NM_031054.2	AGCTCCCGGAAAAGATTGAT	AGTGGCTTGGGGTATCCTCT
MMP-9	NM_031055.1	GTCCAGACCAAGGGTACAGC	AGGGGAGTCCTCGTGGTAGT

Protein extraction and Western blot: Lung tissue was homogenized in lysis buffer (10 mM HEPES, 0.1 mM EGTA, 1 mM DTT, 0.6% NP-40, 10 mM KCl) with adjunction of a protease inhibitor cocktail (0.5 mM PMSF, 5 µg/ml pepstatin, 3 µg/ml aprotinin, 10 µg/ml leupeptin). Fifty µg of protein were then loaded in Laemmli buffer (reducing conditions) on 10% SDS-PAGE gels and transferred on PVDF membranes. Immunodetection was made with primary anti-elastin antibody (ab21610; 1:500) or anti-MMP9 antibody (ab58803; 1:1000) from Abcam, Cambridge, UK. Secondary antibodies were biotinylated anti-mouse or anti-rabbit IgG antibodies from the Peroxidase Vectastain ABC kit (Vector Laboratories Inc., Peterborough, UK), to enhance the signal. Detection was made with Supersignal West Pico Chemiluminescent Substrate (Pierce Protein Research Products, Thermo Fisher Scientific Inc., Rockford, IL, USA) and visualization with LAS-4000 Imager (Fujifilm Life Science, USA).

Standard histology: The right middle lung lobe was washed in PBS, dehydrated in ethanol/xylene series and embedded in paraffin. Sections of 4 µm thickness were stained with either hematoxilin and eosin (H&E) or resorcin-fuchsin for elastin staining.[25]

Synchrotron-radiation X-ray tomographic microscopy: The right upper pulmonary lobes were postfixed with 1% osmium tetroxide (OsO_4) and stained with 4% uranyl nitrate ($UO_2(NO_3)_2$) and mounted as described recently.[26] The X-ray tomographic studies were performed on the TOMCAT beamline at the Swiss Light Source (Paul Scherrer Institut, Villigen, Switzerland). X-ray exposure, conversion to visible light by a YAG:Ce scintillator (18µm thickness, Crismatec Saint-Gobain, Nemours, France), image transformation and 3D-reconstruction were performed as described recently.[27]

Statistical analysis: Statistical comparisons were made using PASW statistics 18.0 from IBM SPSS Inc., Chicago, IL, USA. Results were analyzed by one-way ANOVA followed by Sidak post-hoc tests. Results were considered significant when $P<0.05$.

Results

Physiological parameters: Effects of the applied ventilation parameters on gas exchange were assessed in pilot experiments after one hour of MV. These results, summarized in Table 3, showed that physiologic conditions were maintained for pH and pCO_2, while pO_2 was increased, according to the intended hyperoxemia.

Table 3: Blood Gas Values

Blood gas values (after 1hour of ventilation)	
pO_2 (mm Hg)	212 ± 54
pCO_2 (mm Hg)	50 ± 8
pH	7.38 ± 0.05

Values are expressed as mean ± standard deviation from ≥6 independent animals.

Impact on lung structure: Representative hematoxylin/eosin staining of right middle lung lobe tissue sections are shown in Figure 1. At T:0h, LPS+MV+O_2 animals revealed typical findings of VILI such as an over-distension of distal airspaces, capillary leakage and hemorrhage (Figure 1B). Comparison of CTRL lungs between T:0h and T:48h (Figure 1AC) showed a progression of the alveolarization process with increased secondary septa formation resulting in more numerous and smaller distal air spaces. At T:48h, LPS+MV+O_2 lungs showed a markedly reduced alveolarization represented by fewer and larger air spaces compared to age-matched controls (Figure 1DC). These histological findings were not lobe specific, as assessed by the three-dimensional approach of synchrotron-radiation X-ray-based tomographic microscopy performed on the right upper lobe (Figure 2).

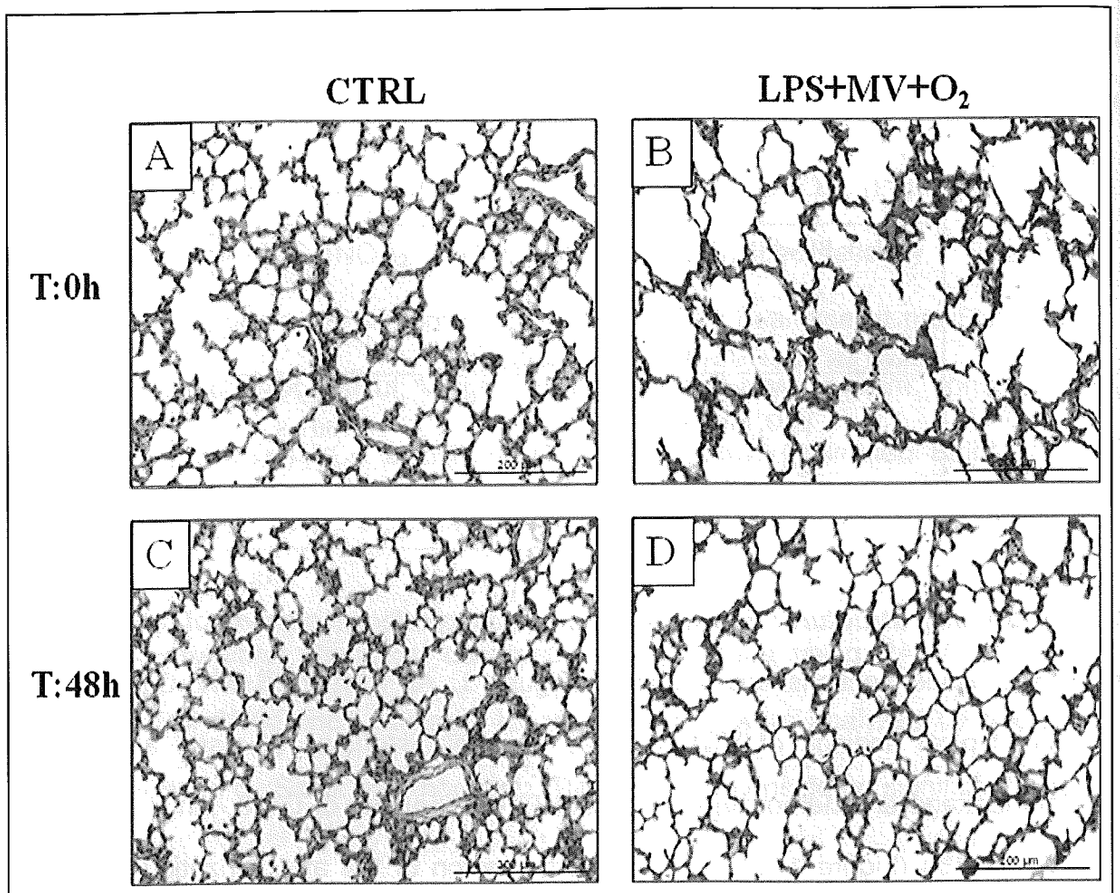

Figure 1:
Conventional histology of newborn rat lungs after mechanical ventilation. Histopathological findings were evaluated by hematoxylin/eosin staining of the right middle pulmonary lobe of newborn rats from age-matched CTRL animals (A, C) and LPS+MV+O_2 animals (B, D) immediately following the intervention (T:0h) (A, B) and 48h thereafter (T:48h) (B, D). Lungs assessed immediately after the intervention (B; n=3)) showed signs of ventilator-induced lung injury with hyperinflation, intra-alveolar hemorrhage and exudates. At T:48h, lungs of control animals (C; n=4)) showed progression of alveolarization with smaller and more numerous alveoli compared to younger controls (A; n=3)). In animals exposed to LPS+MV+O_2 but sacrificed only after an intervention-free interval of 48h (D; n=5)), alveolarization was reduced compared to age-matched controls (C), as evidenced by larger and fewer air spaces.

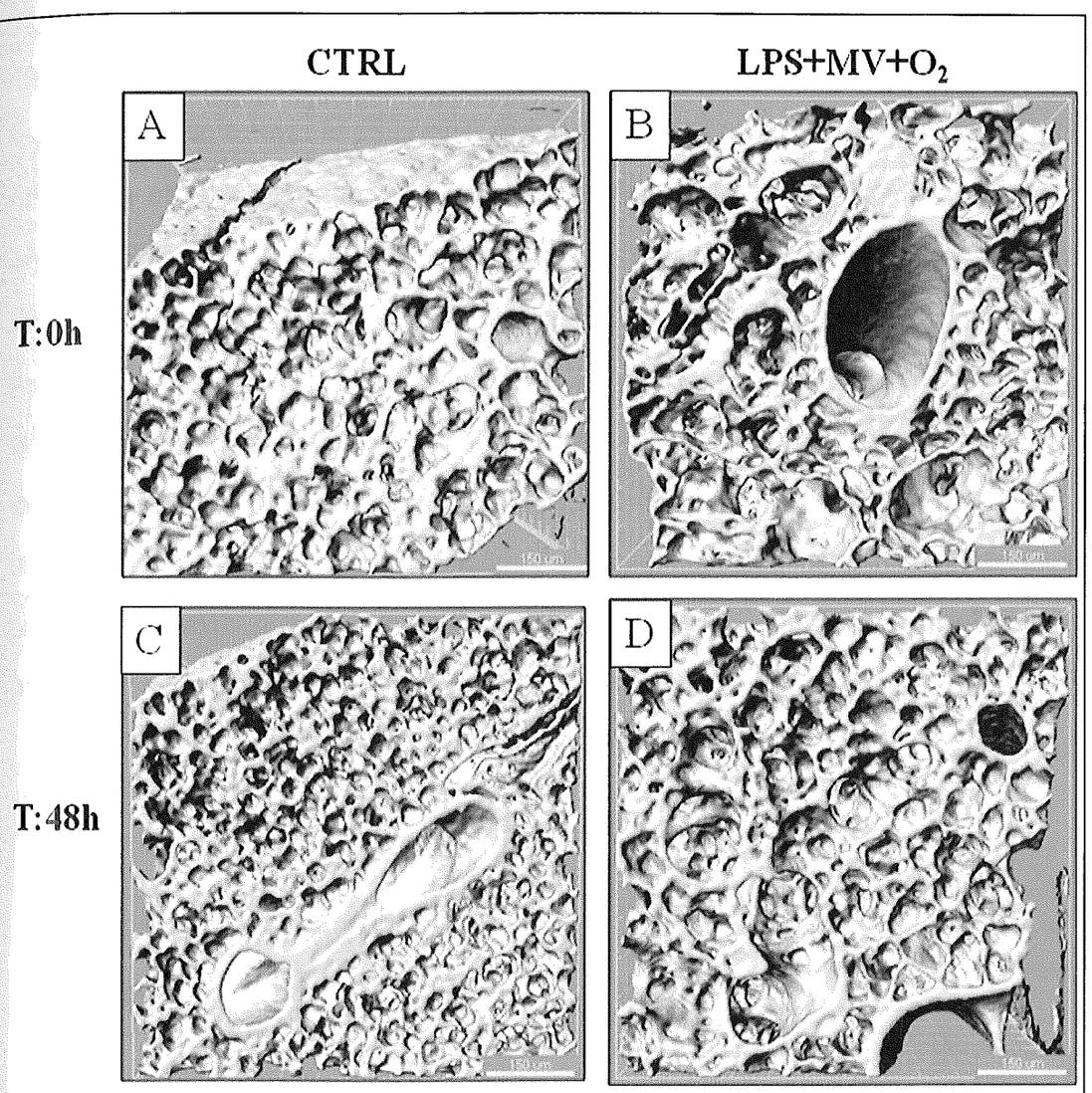

Figure 2:
Synchrotron X-ray tomographic microscopy of newborn rat lungs after mechanical ventilation. Three-dimensional reconstruction of right upper pulmonary lobe morphology was made as a pilot experiment by synchrotron X-ray tomographic microscopy image compilation for LPS+MV+O_2 (B: n=2; D: n=3)) and CTRL (A: n=2, C: n=4) animals at the end of the intervention (A,B) or 48h after (C, D). Lungs directly following the intervention (panel B) showed signs of VILI with hyper-inflated airspaces. With a delay of 48h after mechanical ventilation, lungs showed reduced progression of alveolarization (panel D) compared to controls of the same age (panel C), with fewer and larger airspaces.
Supplementary online videos show 3D-reconstructions of corresponding lung tissue presented in Figure 2 (Video 0h-Ctrl.avi corresponds to panel A; 0h-LPS+MV+O2.avi to panel B; 48h-Ctrl.avi to panel C and 48h-LPS+MV+O2.avi to panel D).

Pro- and anti-inflammatory cytokine gene expression: Treatment effect on pro- and anti-inflammatory cyto-/chemokine gene expression is shown in Figure 3. At T:0h, there was a two-fold increase in all pro-inflammatory cytokine gene expressions (MIP-2, IL-1β, IL-6 and TNFα) in the LPS group. This increase was significant for IL-6 (2.0±0.2 fold change vs CTRL, P=0.002) but below significance for the others. In LPS+MV+O_2 group, there was a further significant increase in MIP-2 gene expression compared to LPS alone (4.1±0.5 fold change vs CTRL, P<0.001 vs CTRL and P=0.002 vs LPS) (Figure 3A). At T:48h, only MIP-2 still showed a small but significant gene expression increase in the LPS+MV+O_2 group compared to controls (2.1±0.5 fold change vs CTRL, P=0.025 vs LPS) (Figure 3B). Anti-inflammatory cytokines IL-10 and IL-1Ra manifested a similar increase in mRNA expression in LPS and LPS+MV+O_2 groups at T:0h which was significant for IL-1Ra (1.9±0.2 and 2.1±0.2 fold change, respectively, P=0.003 and 0.001 vs CTRL) but not for IL-10 (Figure 3C). However, at T:48h, any difference was abrogated (Figure 3D).

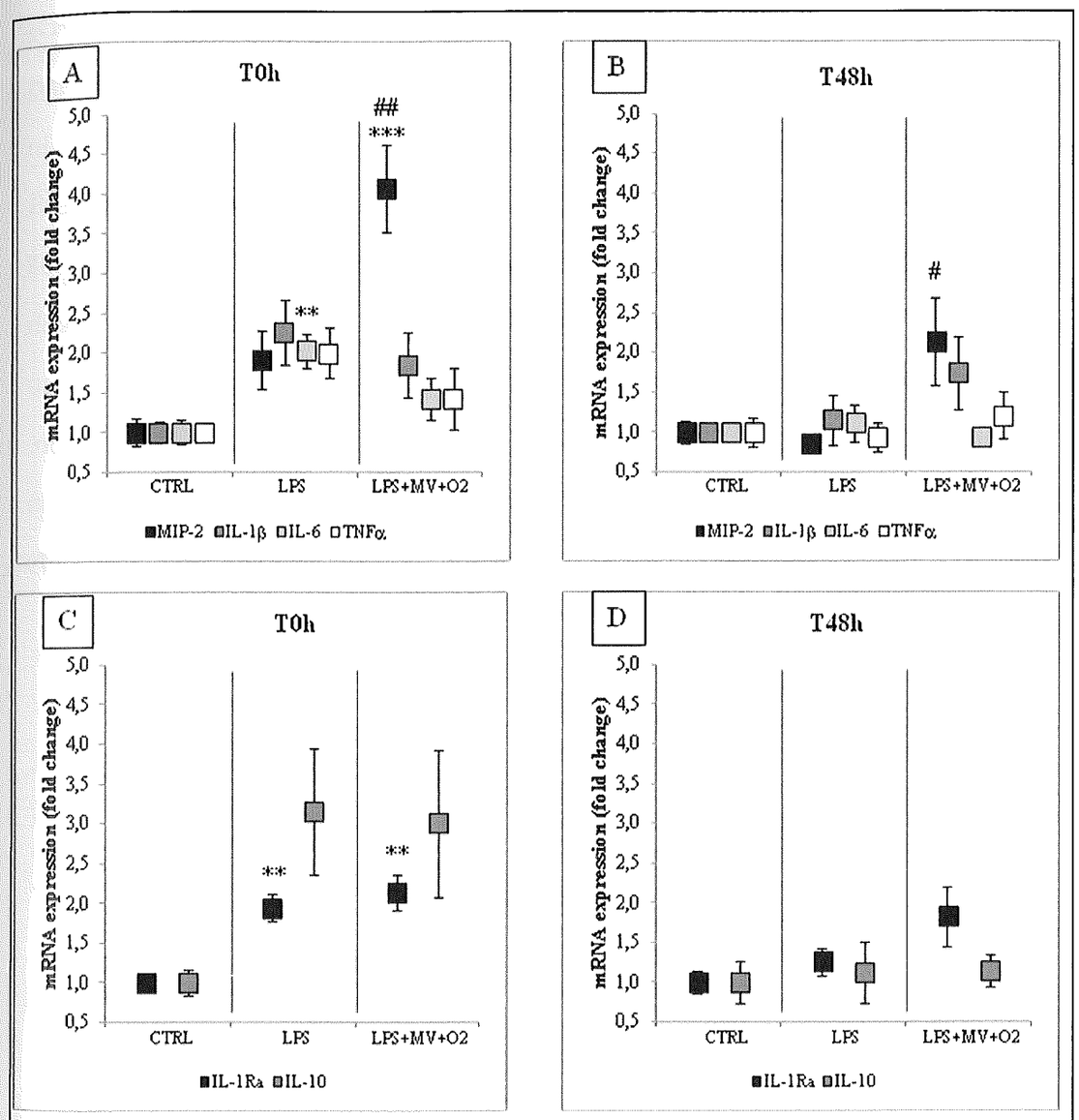

Figure 3:
Pro- and anti-inflammatory cyto-/chemokines genes expression in lungs after mechanical ventilation (MV). mRNA expression of several pro- (A, B) and anti- (C, D) inflammatory cytokines was evaluated by quantitative RT-PCR and normalized by GAPDH directly following the intervention (T:0h) (A, C) or 48h later (T:48h) (B, D). Newborn rat pups were divided into control group, only LPS-injection group or full intervention group (LPS+MV+O_2). At T:0h: CTRL: n=8; LPS+VM+O_2: n=8; LPS: n=10 newborn rat pups per group, and at T:48h: CTRL: n=5; LPS+VM+O_2: n=6; LPS: n=8. Results are expressed as mean ± s.e.m.; **: P< 0.01, ***: P< 0.001 compared to CTRL; #: P<0.05, ##: P<0.01 compared to LPS (by ANOVA and Sidak post-hoc tests).

Matrix metalloproteinases (MMP) expression: MMP-2 gene showed a time-dependent regulation with a decreased expression in both LPS and LPS+MV+O_2 groups at T:0h (0.7±0.1 and 0.6±0.1 fold change vs CTRL, respectively, P=0.003 and P<0.001) which was no longer statistically significant at T:48h (Figure 4A). In contrast, MMP-9 gene expression was slightly but significantly increased due to LPS injection alone at T:0h (2.5±0.3 fold change vs CTRL, P=0.015). In the full intervention group the same trend was observed at T:0h (Figure 4B). At T:48h however, MMP-9 gene expression was further increased in LPS+MV+O_2 group (3.0±0.5 fold change vs CTRL, P=0.007) (Figure 4B). Protein levels at T:48h, assessed by Western blot, evidenced an increase in the latent form of MMP-9 in LPS+MV+O_2 group, compared to CTRL group (49.3 ± 19.6 fold increase, P=0.037) (Figure 4C).

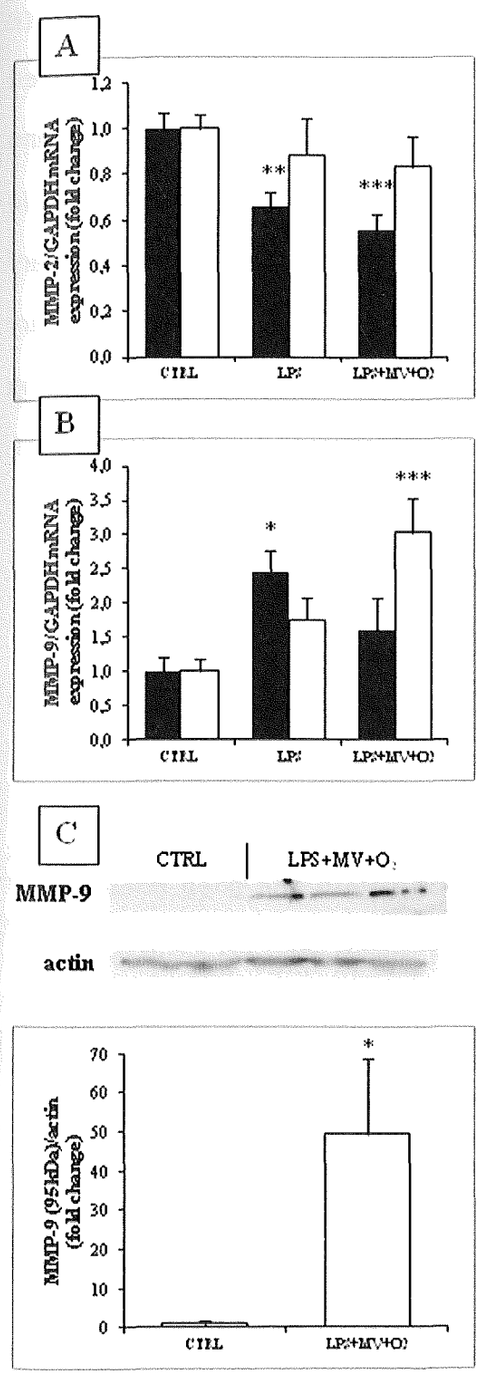

Figure 4:
MMP-2 and MMP-9 gene and protein levels in lungs after mechanical ventilation (MV). mRNA expression of MMP-2 (A) and MMP-9 (B) was evaluated in lung tissue by quantitative RT-PCR and normalized by GAPDH directly following the intervention (T:0h) (black bars) and 48h later (T:48h) (white bars). MMP-9 (95kDa) latent form protein levels were analyzed by Western blot and normalized by actin 48h after the intervention (C). Newborn rat pups were divided into control group, LPS group or full intervention group (LPS+MV+O_2). At T:0h: CTRL: n=8; LPS+VM+O_2: n=8; LPS: n=10 newborn rat pups per group, and at T:48h: CTRL: n=5; LPS+VM+O_2: n=6; LPS: n=8. Results are expressed as mean ± s.e.m.; *: P< 0.05, **: P< 0.01, ***: P<0.001 compared to CTRL (by ANOVA and Sidak post-hoc tests).

Tropoelastin gene expression and histological distribution of elastic fibers: Tropoelastin gene expression also showed a time-dependent regulation, with no difference at T:0h, but a reduced expression at T:48h, in the LPS and LPS+MV+O_2 groups (0.55±0.12 and 0.58±0.11 fold change vs CTRL, overall P=0.046) (Figure 5A). Western blot analyses could not show any modification in protein levels between LPS+MV+O_2 and CTRL individuals at T:48h (Figure 5B) but resorcin-fuchsin staining of lung sections evidenced a modification in elastin distribution (Figure 5CD). In CTRL lungs, elastin fibers were present at the tips of already existing secondary septa and in the walls of large sacculi. Although present at the tips of secondary septa, the specific elastin accumulation in septal walls was almost absent in lung tissue of LPS+MV+O_2 animals.

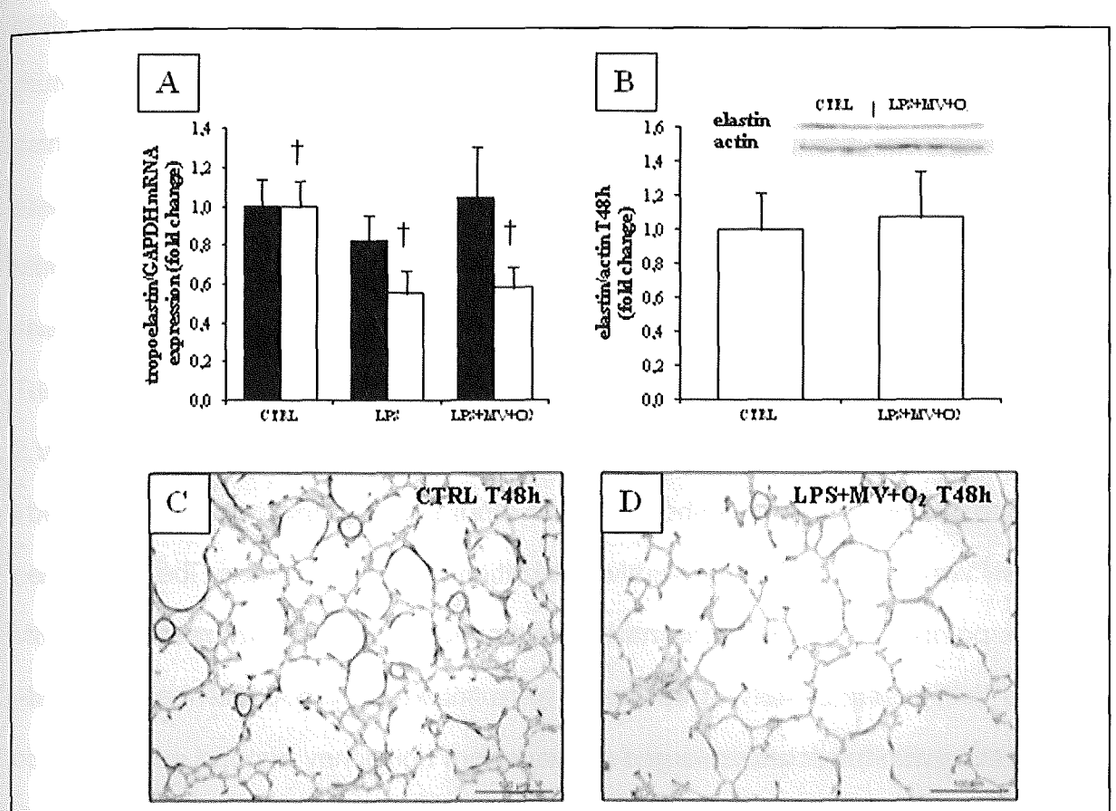

Figure 5:
Tropoelastin gene and protein levels in lungs after mechanical ventilation (MV). mRNA expression of tropoelastin was measured in lung tissue by quantitative RT-PCR directly following the intervention (T:0h) (black bars) and 48h later (T:48h) (white bars) (panel A). Newborn rat pups were divided into control group, LPS group (only LPS injection) or full intervention group (LPS+MV+O_2). Tropoelastin protein levels were analyzed by Western blot and normalized by actin 48h after the intervention (B). Elastin deposition was evidenced by resorcin-fuchsin staining of lung sections at T:48h of controls (C) and after the full intervention (PLS+MV+O_2)(D). At T:0h: CTRL: n=8; LPS+VM+O_2: n=8; LPS: n=10 newborn rat pups per group, and at T:48h: CTRL: n=5; LPS+VM+O_2: n=6; LPS: n=8. Results are expressed as mean ± s.e.m. †: overall P=0.046 (by ANOVA test on the three groups).

Discussion

To evaluate the impact of inflammation, MV and oxygen on immature lungs over time, we tested a newborn rat ventilation model with reversible, non-surgical intubation, in analogy to clinical practices in neonatal intensive care units. Similar intubation procedures have been used in adult rodent models, but to the best of our knowledge, this is the first report of such intubation in newborn rodents.[28] This allowed us to wean off the animals of anesthesia, MV and oxygen after a defined duration of MV, to extubate them to spontaneous respiration, let them recover from the intervention and analyze delayed effects on lung tissue after a ventilation-free interval. While not the prime focus of this work, we first approached lung histology to get insights into the progression of lung structure after MV. Directly following MV, lungs of the intervention groups showed signs of VILI comparable to those described by others.[19] In control animals, the alveolarization process continued between the two time points with ongoing formation of secondary septa, whereas lungs of ventilated animals after 48h of recovery were marked by a pattern of reduced alveolarization with fewer and enlarged airspaces.

MV treatment was shown in different animal models to increase the expression of several cytokines.[16,18] As we intentionally used a rather gentle ventilation strategy with reference to today's clinical approaches, the inflammatory reaction was modest but still significant with increased pro-inflammatory gene expression for MIP-2 (CXCL2, the rodent homologue for IL-8) and a trend towards upregulation of IL-1β, IL-6 and TNFα. Other studies already showed that a more protective ventilation protocol induced a lower increase in circulating IL-6 levels compared to more aggressive ventilation strategies.[29,30] Anti-inflammatory genes like IL-10 and IL-1Ra also showed an increased expression directly following MV, supporting the concept that the organism tries to balance pro- and anti-inflammatory factors. After a 48h recovery period, the pro- and anti-inflammatory response was abrogated, which

revealed that the activation of pro-and anti-inflammatory genes was only a transient event.

We found a distinct gene expression profile for MMP-2 and MMP-9 over time with an early but only transient decrease of MMP-2 gene expression and a delayed increase of MMP-9 at 48h. Both of these proteins are known to be involved in lung development. Whereas MMP-2 is highly expressed during airway branching, MMP-9 expression begins at the saccular stage and predominates during alveolarization.[31] MMP-2 knockout experiments showed that its absence in lungs led to a decreased alveolarization with lower radial alveolar count and larger alveoli at 14 days of age.[32] Tracheal aspirates of preterm infants showed a transient (day 0-3) decrease in MMP-2 levels in patients developing CLD, but MMP-2 levels were shown to be unchanged in lungs of a premature baboon model of longterm MV, compared to the respective controls.[33-36] This controversy could illustrate the more transient character of the MMP-2 gene expression modification observed in our model.

An increased MMP-9/tissue inhibitor of metalloproteinase-1 ratio (TIMP-1) was observed in a baboon ventilation model, as well as in tracheal aspirates and plasma samples of preterm infants developing CLD.[33,35,36] MMP-9 protein levels were shown to be increased in lungs of newborn mice exposed to hyperoxia or in newborns of a transgenic IL-1β model causing a BPD-like disease, but MMP-9 inactivation both relieved and exacerbated the phenotype in these two models, respectively, highlighting the need for further experiments to fully understand the role of MMP-9 in lung injury.[37,38] Nevertheless, in our model, MMP-9 gene and protein levels were increased only at a delay of 48h after treatment. To follow the exact time-course of MMP-9 gene expression after MV might be interesting in the context of the different above-mentioned studies. Additionally, investigating the evolving expression of MMP-9 associated genes might bring new insights in tissue remodeling regulation after MV.

Tropoelastin gene expression and elastin protein deposition in the saccular wall is thought to be a prerequisite for secondary crest formation, as it co-localizes with the uplifting of secondary septa.[39,40] Indeed, elastin knockout mice exhibit abnormal alveolar development.[41] Interestingly, we found no change in tropoelastin gene expression directly following MV, but a 50% decrease after a delay of 48h, which barely reached significance. Several studies have reported a disorganized deposition of elastin fibers in premature infants with chronic lung disease or in animal models of MV.[13,42-44] Histologically, there was an overall reduction in elastin deposition in ventilated animals within the saccular walls which might correspond to the missing prerequisite for further septal formation and alveolarization. However, the amount of lung parenchyma was also decreased due to alveolarization arrest, and therefore the protein levels were unchanged. The discrepancy between mRNA and protein levels could point to changes in elastin metabolism, where several enzymes are involved, either in synthesis (cross-linking) or degradation of elastin. This is emphasized by a recent study in which inhibiting elastase activity enhanced lung growth in ventilated newborn mice and further abrogated MMP-9 activity.[23]

In conclusion, this study demonstrates the feasibility of delayed outcome measurements in newborn rats after a ventilation-free recovery period. The observed differences obtained at the two chosen time points could be summarized as an initial, transitory increase in the inflammation cascade associated to VILI and a secondary, more developmental alteration of lung structure in which proteins like MMP-9 and elastin seem to play a major role. This approach allows the investigation of gene expression and protein production kinetics. Moreover, together with the above-mentioned recent long duration ventilation experiments, it may help to distinguish between effects due to the duration *of* MV versus the delay *since* MV.

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