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The role of inflammation and clotting in the development of neonatal respiratory distress syndrome

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**The role of inflammation and clotting in the
development of neonatal respiratory distress
syndrome.**

Anneke Jaarsma

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development of neonatal respiratory distress
syndrome.**

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**Harde gegevens voeden het verstand,
zachte gegevens zorgen voor wijsheid.**

Mintzberg, Ahlstrand, Lampel
Op strategiesafari

The role of inflammation and clotting in the development of neonatal respiratory distress syndrome.

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Nederlandse samenvatting.

Activatie van ontsteking en stolling bij de ontwikkeling van neonatale RDS.

Activatie van ontsteking en stolling bij de ontwikkeling van neonatale RDS.

Longen hebben als functie te zorgen voor gaswisseling. Gaswisseling is het proces in de long waarbij zuurstof uit de omgevingslucht in het bloed komt, en koolzuur, dat in het lichaam is geproduceerd, uit het bloed wordt verwijderd. Als gaswisseling onvoldoende plaats vindt, ontstaat zuurstoftekort en koolzuurophoping in het lichaam. Om gaswisseling adequaat te laten plaatsvinden moeten de longen bij elke inademing goed ontplooid worden. Bij elke uitademing valt de long weer iets samen, maar het is belangrijk dat longblaasjes (alveoli) niet geheel samenvallen, want er is meer kracht nodig om volledig dichtgevallen alveoli te openen dan deels opengebleven alveoli. Hiervoor is de aanwezigheid van surfactant belangrijk. Surfactant is een dun laagje dat aanwezig is op de scheidingslijn tussen lucht en water aan de binnenzijde van de alveoli. Surfactant heeft een oppervlaktespanning verlagende werking zoals in een zeepbel, waardoor de alveoli niet samenvallen bij uitademing.

Neonatal Respiratory Distress Syndrome (neonatale RDS) is een longaandoening van te vroeg geboren kinderen. Deze aandoening wordt veroorzaakt doordat de longen nog niet volledig ontwikkeld zijn. Naast structurele onrijpheid wordt ook onvoldoende surfactant gemaakt. Hierdoor hebben de alveoli sterk de neiging samen te vallen en kost elke inademing erg veel kracht. Vaak hebben te vroeg geboren kinderen de kracht niet om de ademhalingsbewegingen lang vol te houden omdat ze door hun vroeggeboorte relatief weinig spierweefsel hebben. Ze kunnen de gaswisseling hierdoor onvoldoende waarborgen en ontwikkelen zuurstoftekort en koolzuurophoping. De behandeling die hiervoor wordt ingesteld is kunstmatige beademing en surfactant toediening.

Soms is het niet mogelijk de te vroeg geboren kinderen zo te beademen dat voldoende zuurstof in het bloed komt en voldoende koolzuur wordt verwijderd en overlijden ze. Andere kinderen kunnen weliswaar beademd worden, maar hebben hoge beademingsdrukken en veel zuurstof nodig. De longen kunnen hierdoor beschadigd raken met als gevolg een verminderde longfunctie: men spreekt dan van chronische longschade of bronchopulmonale dysplasie.

Bij elke beschadiging doet het lichaam moeite om die schade weer te herstellen. Er ontstaat een “opruimreactie” die te vergelijken is met een afweerreactie tegen bacteriën of virussen die ook dreigen het lichaam schade toe te brengen: de zgn. ontstekingsreactie.

Ook in de longen van te vroeg geboren met longproblemen lijkt een ontstekingsreactie te ontstaan. Bij kinderen die op latere leeftijd overlijden aan de gevolgen van bronchopulmonale dysplasie zijn bij onderzoek van het longweefsel tekenen gevonden van deze ontstekingsreactie. Dit in tegenstelling tot onderzoek van longweefsel van kinderen met gezonde longen die aan een andere ziekte overlijden waarbij het geen tekenen van ontstekingsreactie worden gevonden. Het lijkt dus alsof de ontstekingsreactie bij bronchopulmonale dysplasie geen gunstig effect heeft of dat de beschadiging bij de kinderen die overlijden dusdanig ernstig is dat er geen herstel meer mogelijk is. Om te onderzoeken of de ontstekingsreactie een adequate, maar tekort schietende reactie is op beschadiging of een onvoldoende gereguleerde reactie met schadelijke effecten, is

onderzocht wanneer de reactie begint en hoe het verloop is. Kinderen met neonatale RDS blijken al snel na de geboorte tekenen van een ontstekingsreactie in de longen te hebben. Bij de kinderen die herstellen van de ademhalingsproblemen komt de ontstekingsreactie tot rust, bij de kinderen die bronchopulmonale dysplasie ontwikkelen, blijft de ontstekingsreactie bestaan. Het is niet bekend of de gevonden vroege ontstekingsreactie beschouwd moet worden als een ongunstige reactie omdat er extra schade door ontstaat, of juist als een gunstige reactie die leidt tot herstel. Het lijkt wel belangrijk dat de ontstekingsreactie op tijd tot rust komt om bronchopulmonale dysplasie te voorkomen. Als de vroege ontstekingsreactie leidt tot ernstiger ademhalingsproblemen ligt het voor de hand te proberen deze reactie in te dammen, om op deze manier zowel ademhalingsproblemen te voorkomen als een chronische ontstekingsreactie te beperken die kan leiden tot bronchopulmonale dysplasie.

Naast teken van een ontstekingsreactie speelt ook activatie van de stollingscascade een rol bij de symptomen en mogelijk het ontstaan van neonatale RDS. Dit is gebleken uit bloedonderzoek bij kinderen met neonatale RDS en uit microscopisch onderzoek van longweefsel van kinderen die zijn overleden aan neonatale RDS. De ontstekingsreactie en de stollingsactivatie kunnen elkaar onderling beïnvloeden via uitgebreide regelmechanismen. Als de één geactiveerd wordt zal de ander via onderlinge signalen ook geactiveerd worden. Welke van de twee bij neonatale RDS als eerste geactiveerd wordt is niet bekend.

Wij hebben onderzoek gedaan naar het exacte moment waarop de ontstekingsreactie begint en stollingsactivatie plaatsvindt en wat de relatie met de ademhalingsproblemen is. Dit onderzoek is uitgevoerd bij lammeren die via een keizersnee geboren werden om ze te vroeg geboren te kunnen laten worden. Lammeren die te vroeg geboren worden vertonen dezelfde ademhalingsproblemen als te vroeg geboren kinderen. De lammeren zijn net als te vroeg geboren kinderen beademd en bloed en materiaal uit de longen zijn afgenomen om naar de ontstekingsreactie te kijken.

In dit proefschrift wordt beschreven dat een ontstekingsreactie 15 minuten na de geboorte al kan worden aangetoond. Stollingsactivatie vindt iets later plaats zodat geconcludeerd wordt dat stollingsactivatie het gevolg is van de ontstekingsreactie en niet de oorzaak. In het vervolgonderzoek is de stollingsactivatie dus veel minder belangrijk. “Gewone” kunstmatige beademing waarbij steeds lucht in de longen geblazen wordt, is vergeleken met hoog frequente beademing die mogelijk minder beschadigend is. Bij de hoogfrequente beademing blijven de alveoli steeds volledig ontplooid en wordt de lucht er als het ware in- en uitgetrild. Uit het onderzoek blijkt dat er wat betreft de ernst van de ademhalingsproblemen en de ernst van de ontstekingsreactie geen verschil is tussen de 2 beademingsvormen.

Omdat in de eerste studie het eerste bloedmonster 15 minuten na de geboorte al tekenen van een ontstekingsreactie liet zien is vervolgens onderzocht of het exacte moment van activatie kan worden gevonden als nog vroeger bloedmonsters worden afgenomen. In dit vervolgonderzoek wordt het eerste bloedmonster voor het doorknippen van de navelstreng afgenomen en wordt vervolgens elke 5 minuten bloed afgenomen. Het blijkt nu dat de ontstekingsreactie al binnen enkele minuten na de geboorte optreedt. Om na te gaan of de

ontstekingsreactie uniek is voor de te vroeg geborene hebben wij het onderzoek op gelijke wijze herhaald bij voldragen lammeren. Bij deze dieren, die geen ademhalingsproblemen vertonen zien wij dat de ontstekingsreactie veel minder heftig verloopt en ook sneller stopt. Hiermee is de relatie tussen de ontstekingsreactie en het ontstaan van ademhalingsproblemen direct na de geboorte minder waarschijnlijk geworden en lijkt de hele vroege ontstekingsreactie meer gerelateerd aan de (vroeg)geboorte zelf.

Om ademhalingsproblemen ten gevolge van vroeggeboorte te voorkomen wordt tegenwoordig aan de zwangere vrouw in de kliniek betamethason toegediend. Dit is een hormoonpreparaat waarvan bekend is dat het de longen van het kind sneller doet rijpen en de surfactant productie verbetert. Een bijkomende werking van betamethason is dat het ook de ontstekingsreactie remt. Betamethason is aan de schapen gegeven 24 uur voor de keizersnee en gekeken is wat het effect hiervan is op de ontstekingsreactie en de ademhalingsproblemen bij het lam. Na betamethason toediening blijkt bij het lam in het geheel geen ontstekingsreactie meer te kunnen worden aangetoond. De ademhalingsproblemen zijn klinisch minder ernstig. Hiermee lijkt het logisch te veronderstellen dat er toch een relatie is tussen de ontstekingsreactie en de ademhalingsproblemen direct na de geboorte. Echter, betamethason bevordert ook de longrijping en vermindert de hoeveelheid vocht in de longen. Dit zijn beide bekende factoren die ook tot minder ademhalingsproblemen leiden.

Om te zien wat het precieze effect van de ontstekingsreactie op het uiteindelijke longweefsel zelf is, is microscopisch onderzoek van longweefsel gedaan van de lammeren nadat ze 8 uur beademd waren. Zowel de effecten van betamethasontoediening aan de schapen als effecten van toediening van surfactant aan de lammeren zijn bestudeerd. Van toediening van surfactant is bekend dat het de ademhalingsproblemen vermindert bij te vroeg geboren kinderen (en ook bij te vroeg geboren lammeren). Na toediening van betamethason aan de schapen worden minder tekenen van ontsteking in het bloed van de lammeren en nooit enige ontstekingsreactie in de longen aangetoond, maar de microscopische longbeschadigingen nemen niet af. Toediening van surfactant direct na de geboorte leidt niet tot beïnvloeding van de ontstekingsreactie in bloed en longen en ook bij microscopisch onderzoek zijn de longbeschadigingen net zo ernstig als zonder surfactant.

We hebben in onze studies gevonden dat de ontstekingsreactie die vroeg na de geboorte optreedt mogelijk heftiger verloopt wanneer lammeren (en kinderen) te vroeg geboren worden en beademd moeten worden, maar dat dit geen relatie lijkt te hebben met de ernst van ademhalingsproblemen ten gevolge van longonrijpheid en surfactant tekort. Betamethason toediening voor de geboorte aan de moeders remt de ontstekingsreactie, maar vermindert de longbeschadiging zelf niet. Misschien vermindert betamethason enerzijds wel de ademhalingsproblemen door de snellere longrijping en is minder beademing nodig waardoor er geen bijkomende longbeschadiging optreedt, maar is anderzijds mogelijk dat het ontbreken van een adequate ontstekingsreactie bij doorgaande beschadiging (b.v. beademing) leidt tot onvoldoende “opruimreactie” en daardoor tot bronchopulmonale dysplasie.

Op grond van onze onderzoeksresultaten in combinatie met literatuurgegevens concluderen wij dat de door ons beschreven ontstekingsreactie in de longen geen relatie heeft met de ademhalingsproblemen direct na de geboorte. Er zijn echter aanwijzingen dat, indien een ontstekingsreactie op gang gekomen is, door welke oorzaak dan ook, er een verhoogde kans op chronische longziekte op de langere termijn bestaat.

Het lijkt dus zaak een ontstekingsreactie af te zwakken om op de langere termijn bronchopulmonale dysplasie te voorkomen. Dit kan door infecties te proberen te vermijden, maar ook door een beademingsvorm toe te passen die zo weinig mogelijk activatie veroorzaakt. Een geactiveerde ontstekingsreactie kan afgeremd worden door medicijnen, bijvoorbeeld dexamethason (vergelijkbaar met betamethason), maar misschien selectiever door anti-ontstekingsmiddelen zoals ibuprofen of indomethacine. Een ander alternatief voor de toekomst is ingrijpen in de ontstekingsreactie zelf of in de regulatiemechanismen die het lichaam heeft om een ontstekingsreactie weer tot rust te brengen. Gevaar is echter altijd dat de delicate balans tussen excessieve ontsteking met beschadiging als gevolg en insufficiënte ontsteking, waardoor onvoldoende afweer tegen beschadiging en infecties, verstoord raakt. Verder onderzoek zal plaats moeten vinden om het juiste evenwicht te vinden.

Chapter 1

General introduction.

General introduction.

Although neonatal intensive care has improved dramatically the last two decades and treatment has become possible for extreme preterm infants, Respiratory Distress Syndrome of the neonate (neonatal RDS) is still an important problem in treatment of preterm infants. Neonatal RDS is a disease in preterm infants, characterized by structural immaturity of the lungs, surfactant deficiency and abnormal surfactant function. New therapies are developed, aiming at treatment of these problems and include endotracheal surfactant administration and antenatal glucocorticoid administration.^[1-3] Other therapeutic strategies aim at reducing ongoing damaging effects of mechanical ventilation and exposure to high oxygen concentrations on the immature lung. In this respect high frequency ventilation is elaborated clinically and treatment with oxygen radical scavengers is under investigation.^[4-9] In *chapter 2* an overview of pathophysiology and treatment of neonatal RDS is given.

Although surfactant replacement has lowered mortality and morbidity among preterm infants with neonatal RDS, some infants do not improve following surfactant replacement therapy, while others improve only for a short period or develop chronic lung disease. In the lungs of infants dying of acute neonatal RDS or dying of chronic lung disease (or bronchopulmonary dysplasia), protein rich edema and several inflammatory mediators have been found.^[10,11] Therefore it is concluded that inflammation plays an important role in the pathogenesis of neonatal RDS or in the pathogenesis of the development of chronic lung disease.^[12-14] In neonatal RDS and chronic lung disease, not only local (lungs), but also systemic activation of the inflammatory reaction and clotting can be found, similar as in Adult Respiratory Distress Syndrome.^[15-20] In *chapter 3* an overview is provided of recent literature about the role of inflammation in neonatal RDS.

Aim of the thesis.

The aim of this thesis is to explore the role of local and systemic activation of inflammation and clotting in the pathogenesis and the severity of neonatal RDS. We have investigated these systems and their mutual interactions in an animal model. In the model we have studied the exact moment of activation of the inflammatory reaction and the relationship between the activation of the inflammatory reaction and the severity of the respiratory problems. We also have studied the impact of different ventilatory modes (conventional ventilation: CV) and high frequency oscillatory ventilation (HFOV) on activation of the inflammatory reaction. In addition we have studied the effects of already established therapies as endotracheal surfactant administration and antenatal glucocorticoid administration on activation of the inflammatory reaction with respect to the development of respiratory distress.

Introduction to the studies.

In 1997 we started the studies in preterm lambs. To control for gestational age, all lambs were born by cesarean section at 132 days gestational age (term 145 days). All lambs were ventilated for severe neonatal RDS.

Based on experimental studies, HFOV is considered to be less injuring to the preterm lungs than CV.^[4,5,21] Lung injury leads to activation of the inflammatory reaction, so we expect HFOV ventilated lambs to have an attenuated activation of the inflammatory reaction compared to CV ventilated lambs. So CV ventilated lambs have been compared to HFOV ventilated preterm lambs with respect to measurements of activation of the inflammatory reaction and clotting. In *chapter 4* this study has been described. Blood and bronchoalveolar lavage fluid have been sampled from 15 minutes after delivery till 8 hours after delivery. In this study already at 15 minutes after delivery, the inflammatory reaction has been demonstrated to be activated. The next study has the question whether activation of the inflammatory reaction and clotting could be found immediately, in fact within minutes, after delivery. In *chapter 5* the results of the study in which parameters of activation of the inflammatory reaction before delivery and in the first 15 minutes after delivery have been studied, are described. Ventilated preterm lambs with neonatal RDS have been compared to spontaneously born and spontaneously breathing term lambs to investigate whether it is neonatal RDS and mechanical ventilation that activate the inflammatory reaction or birth itself.

In recent years antenatal glucocorticoid administration to pregnant mothers who were expected to deliver preterm infants < 32 weeks of gestational age within 1 to 7 days, proved to be effective in reducing severity of neonatal RDS.^[2,3] Antenatal glucocorticoids are known to accelerate lung maturity and surfactant production and are effective in promoting fluid clearance from the lungs immediately after delivery, resulting in improved lung function after delivery.^[22,23] Because of the immunomodulatory effects of glucocorticoids, antenatal glucocorticoid administration can be expected to attenuate the activation of the inflammatory reaction and clotting immediately after delivery that we have demonstrated previously. In *chapter 6* the results of this study are described.

In the final study, we have investigated whether the activation of the inflammatory reaction is related to the development of RDS, clinically as well as histologically. Endotracheal surfactant administration and antenatal glucocorticoid administration have beneficial effects on the development of RDS, so we have studied the effects of those two therapies on the activation of the inflammatory reaction in relationship to the development of RDS. Histologically, neonatal RDS is characterized by irregular patterns of alveolar collapse and hyperinflation.^[24] It is expected that attenuation of the inflammatory reaction will improve histologic findings if the inflammatory reaction is related to the development of RDS. In *chapter 7* the results of this study are described.

In the general discussion of this thesis, *chapter 8*, the implications of the results of the studies we performed are discussed.

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Chapter 2

Neonatal respiratory distress syndrome.

Neonatal respiratory distress syndrome.

Introduction.

Neonatal respiratory distress syndrome (neonatal RDS), previously called hyaline membrane disease, is a developmental disorder of mainly preterm infants. Structural immaturity of the lungs, surfactant deficiency and surfactant dysfunction, are the main problems of the preterm newborn, leading to respiratory distress. Despite of new preventive strategies, neonatal RDS is still the leading cause of mortality and morbidity in neonatal intensive care.^[1] The incidence and severity of neonatal RDS show an inverse relationship with gestational age. Approximately 50% of infants born after a gestational age of 25-27 weeks develop neonatal RDS whereas fewer than 20% of preterm infants born at a gestational age of 31 weeks develop the disorder and the incidence decreases to 1% for infants born term.^[1]

Etiology/pathophysiology.

Although prematurity is the main risk factor to develop neonatal RDS, additional risk factors are: male sex, white race, twin pregnancy, maternal diabetes, maternal pre-eclampsia, delivery by cesarean section and prenatal and perinatal asphyxia.^[1]

Central in the pathogenesis of neonatal RDS are structural pulmonary immaturity and immaturity of the surfactant metabolism.

Structural pulmonary immaturity can be expected after preterm birth, because normal lung development occurs throughout pregnancy and continues after birth till 2 to 3 years of age.^[1,2] During the embryonic phase (0-7 weeks) the lung bud, stemming from the embryonic foregut, undergoes repetitive branching to form the proximal structures of the tracheo-bronchial tree. In the following pseudoglandular phase (7-17 weeks) branching of the airways and concomitant blood vessels continues. The forming of acinar structures (respiratory bronchioli, alveolar ducts and primitive alveoli) takes place in the canalicular phase (17-27 weeks). Only after 27 weeks of gestational age, in the saccular phase (28-36 weeks), the peripheral airways enlarge and the gas-exchanging surface enlarges by thinning of the arterial septa. The process of formation of definitive alveoli continues from 36 weeks gestational age till 2 to 3 years after birth. From this developmental process one can understand that preterm infants have structural immature lungs, characterized by a small number of alveoli or no alveoli at all but sacculi, which have thick epithelium and septa where pulmonary vessels have not penetrated yet. Therefore structural pulmonary immaturity leads to impaired diffusion of oxygen and carbon dioxide, which leads, among others, to the clinical characteristics of neonatal RDS.

In preterm infants the surfactant metabolism is also immature. In the embryonic phase, undifferentiated columnar epithelium lines the airways. During the canalicular phase differentiation of the epithelium in flat alveolar type I cells, which line the alveolar surface, and cuboid type II cells takes place. From 20 weeks of gestational age the type II cells contain lamellar bodies which are the intracellular storage places of surfactant. Surfactant contains 70-80% phospholipids, about 10% protein and 10% neutral lipids. The main phospholipid is phosphatidylcholine, in saturated form it is the most important surface-active component of surfactant.^[3] Therefore phosphatidylcholine concentration in amniotic fluid is used as marker for the amount of available active surfactant. During the second half of gestation synthesis of surfactant increases linearly.^[4] Figure 1 shows the relationship between gestational age and phosphatidylcholine content of the lung tissue: the amount of surfactant is linearly related to gestational age.^[3] From about 35 weeks of gestational age surfactant is released to the alveoli (figure 1) by exocytosis from the type II cells and forms, after unraveling to tubular myelin, a monolayer on the air-liquid interface.

Figure 1.
Concentrations of saturated phosphatidylcholine found in lung tissue and in alveolar samples expressed versus percentage gestational age (adapted from Clements JA and Tooley WH).

Catecholamines, calcium and alveolar stretch stimulate release of surfactant.^[5] In term newborns an active re-uptake and recycling process of the secreted surfactant takes place. Surfactant stabilizes the alveoli by decreasing the surface tension at the air-liquid interface inside the alveoli and it plays an important role in the water clearance from the alveoli. Another function of surfactant lies in the host defense of the lungs.^[6-8]

Surfactant deficiency will therefore lead to alveolar collapse and atelectasis. Alveolar collapse leads to distension of the proximal airways because they have a higher compliance. Overdistension of the proximal airways injures the epithelium and causes necrosis of the airway epithelium and desquamation. The resulting epithelial lesions allow leakage of proteins into the small airways and alveolar space. Intra-alveolar proteins interfere with the formation of the surfactant monolayer and can interfere with the biophysical activities of surfactant.^[9,10] This can lead to further alveolar collapse and atelectasis. Furthermore, the

protein leakage also causes an increased osmotic pressure in the alveolar spaces, attracting fluids or interfering with the normal fluid clearance from the alveoli which will result in protein rich edema in the alveoli.

Therefore, neonatal RDS is a disease characterized by hampered diffusion, alveolar collapse, and pulmonary edema.

Clinical manifestations.

Understanding the pathophysiology of neonatal RDS, one can explain the typical clinical signs. Alveolar collapse results in a decrease of functional residual capacity and leads to cyanosis of the patient. Progressive alveolar collapse also causes a decrease of compliance of the lung. Clinically intercostal retractions and tachypnea can appreciate this as the infant tries to compensate for a low tidal volume and tries to maintain an adequate minute volume. Expiratory grunting is an effort of the infant to avoid alveolar collapse. The vocal cords are simultaneously closed, so that a resistance of the expiratory flow is created that is counteracting alveolar collapse.

The chest X-ray of infants suffering from neonatal RDS is characterized by a typical reticulogranular pattern with an air bronchogram, due to collapsed alveoli and distended gas filled larger airways.^[11] The more opaque the X-ray the more severe the RDS.

Typical for neonatal RDS is that the clinical signs develop gradually in the first hours after birth. It is assumed that breathing movements of the surfactant deficient lung generate shear forces to the epithelium of the terminal bronchioli, resulting in epithelial lesions. Subsequently fibrinogen and other serum proteins leak through the epithelial lesions into the alveolar space. These proteins inhibit surfactant function and hence cause progressive alveolar collapse. Depending on the severity of the disease, respiratory failure occurs and artificial ventilation is indicated. When the natural course proceeds uncomplicated, respiratory signs diminish from the second to third day after birth leading to recovery.^[12,13] However, complicated RDS will prolong the infant's need for ventilatory support with high oxygen concentrations and high inspiratory pressure and may lead to chronic lung disease and prolonged respiratory support.^[14]

Treatment.

The treatment of neonatal RDS consists of general supportive care and specific treatment that includes respiratory support for developing hypoxemia and hypercapnia and the endotracheal supplementation of surfactant.

General supportive care.

The initial stabilization of the infant is critical; the smaller the infant the more important this becomes. The infant should not be allowed to become hypothermic, hypoglycemic or hypovolemic. Hypovolemia can be avoided by not clamping the umbilical cord immediately after delivery. In this way the risk of metabolic acidosis is reduced and hence the risk of pulmonary vasoconstriction. Fluid administration is to be directed to meet insensible water losses and urine production.

Specific treatment.

In mild cases with cyanosis as the only symptom of neonatal RDS, predominantly in neonates born after a gestation of 32-36 weeks, the administration of supplementary oxygen is sufficient to restore hypoxemia. When the infant needs more than 30-40% oxygen to obtain adequate oxygenation generally more features of neonatal RDS occur: tachypnea, intercostal retractions, grunting and nasal flaring. Then the application of nasal Continuous Positive Airway Pressure (CPAP) is indicated. Gregory et al introduced this therapy for neonates with RDS in 1971.^[15] Oxygenation is improved by an increase of the functional residual capacity of the lung, through recruitment of collapsed alveoli. CPAP prevents ongoing alveolar collapse and in this way shear forces to the terminal bronchioli are limited. Consequently less serum proteins, that inhibit surfactant function, are permitted to enter the alveolar space.^[12] A recent study indicates that continuous positive airway pressure leads to less activation of inflammation in laboratory animals with neonatal RDS compared to the use of no positive airway pressure.^[16] Thus CPAP is a rational tool to attenuate the progression to severe neonatal RDS.

Infants born after a gestational age of less than 30 weeks are prone to develop severe neonatal RDS and as the gestation is shorter, the risk for respiratory failure is higher. For those patients mechanical ventilation is indicated. This technique was initiated in the early years 1950. Introduction of polyvinyl nasotracheal tubes by Brandstadter in 1962 eliminated the need for tracheotomy and made mechanical ventilation a more feasible therapy.^[17] In this first era of mechanical ventilation of newborns, modified adult ventilators were used, applying high airway pressure and gas flow. Survival improved but mortality remained high compared to the numbers that are noticed presently. In the 1980s it was observed that patients with neonatal RDS made spontaneous breathing movements while they were mechanically ventilated.^[18] It was noted that infants that actively expired against positive pressure inflations developed pneumothoraces frequently.^[19] The idea of synchronization of mechanical ventilation with the spontaneous breathing movements of the patient emerged. It took until 1986 when a successful device was developed by Mehta et al. who used the signal of a respiration monitor to trigger the ventilator.^[20] Since that time most ventilators are equipped with the mode synchronized intermittent positive pressure ventilation. Despite improvement of the outcome of patients with neonatal RDS, it was observed that “ventilator induced lung injury” remained a significant problem. The concept that lung injury was caused

by high airway pressure was rejected by animal studies that showed that the large changes of volume, and not the pressure swings, were responsible for the injuries.^[21,22] Consequently most neonatologists nowadays try to control gas exchange of their patients with lower tidal volumes.

Further elaborating on the concept that large volume changes are harmful to the lung a new mode of mechanical ventilation was introduced: high frequency ventilation (HFV).^[23,24] Many devices are available, but in Europe two types of HFV are used: High Frequency Oscillatory Ventilation (HFOV) and High Frequency Flow Interruption (HFFI). The principle of HFV is the generation of a pressure wave at a supra physiological rate by an electromagnet driven diaphragm (HFOV) or a microprocessor controlled solenoid superimposed on the mean airway pressure. The opening and closing of the solenoid generates a pulse of high-velocity gas, which is transmitted down the airways. HFV proved to be very efficient in the oxygenation and CO₂ removal of patients with neonatal RDS. Since the introduction of HFV many animal studies have been published, demonstrating that this mode of ventilation leads to better lung inflation and less alveolar and airway damage than does conventional ventilation.^[25,26] Despite the beneficial effects that were found in animal studies, prospective randomized clinical trials in patients with neonatal RDS have yielded inconsistent results. As investigators understood the importance of applying a “high-volume” strategy, the results improved in terms of lower incidence of chronic lung disease. So far it can not be stated that HFV is superior to conventional ventilation in the optimal ventilation of infants with RDS.

The most important development in the treatment of neonatal RDS is the endotracheal instillation of surfactant. Since the discovery of Avery and Mead that surfactant deficiency was a key factor in the pathogenesis of RDS, many investigators were stimulated to find ways to supplement this missing compound in the lung.^[27] After promising results in animal experiments, it was Fujiwara et al. who presented the first series of patients that were treated with surfactant administration endotracheally.^[28] In the years 1980 a large number of randomized clinical trials have been published. The majority of these studies indicate that mortality of infants, who are treated with surfactant, is reduced significantly. The impact on the development of chronic lung disease is less clear. This therapy has become routine treatment for neonatal RDS in developed countries all over the world.

Development of chronic lung disease, bronchopulmonary dysplasia.

Most infants recover from neonatal RDS. However some infants develop chronic lung disease or bronchopulmonary dysplasia (BPD).

In 1967 Northway described BPD as severe lung injury.^[29] Persistent tachypnea, auscultatory rales and retractions and requirement for supplemental oxygen for more than 28 days in an infant that was ventilated for respiratory distress in the first week of life clinically characterize the disease. Radiological characteristics are persistent strands of density in both

lungs, alternating with areas of normal or increased lucency.^[30] Histologic examinations of infants dying of severe BPD show marked necrosis of airway epithelium, resulting in intraluminal accumulation of cell rests, mucus and inflammatory cells. Depending on the severity and the stage of BPD, exudates with fibroblasts and newly formed connective tissue elements reflecting repair mechanisms can be found or severe peribronchial fibrosis, obliterative bronchiolitis and severe derangement of the alveolar architecture showing a lung which is damaged beyond repair.^[31]

Mechanical ventilation and oxygen exposure on an immature lung are considered risk factors for the development of BPD.^[32] Histologically, lungs of infants with RDS and BPD are characterized by irregular inflation. Alveoli and airways with high compliance accompany alveoli and airways with low compliance. As well overdistension as atelectasis are both consequences and causes of lung injury. Mechanical ventilation was initially considered to cause lung injury because of positive pressure on the lungs leading to overdistension of the alveoli. This was the concept of barotrauma.^[33-35] However, it is merely stretch of the alveoli due to large tidal volume breaths by mechanical ventilation that causes damage to the pulmonary capillary endothelium, alveolar and airway epithelium and the basement membranes. This is merely the concept of volutrauma.^[36-39]

Atelectrauma is the consequence of loss of alveolar recruitment. Due to surfactant deficiency the alveoli are prone to collapse and overdistension of the proximal airways. This will lead to epithelial injury. The epithelial lesions allow leakage of proteins into the small airways and alveolar space, which interfere with normal surfactant function.^[9,10] This can lead to further alveolar collapse and atelectasis.

In the years after 1990, BPD is not the same as Northway once described. With progress in neonatal care, including antenatal glucocorticoids, surfactant and new ventilatory strategies, the preterm infants born after a gestational age of 28 weeks, less frequently develop BPD. The new infants with BPD are born at gestational ages of 24-28 weeks, are less than 1000 grams and they don't always suffer from severe neonatal RDS soon after birth, they sometimes don't even need ventilation after birth. Most of them are treated with antenatal corticosteroids for lung maturation. In Northway's days these infants died because of limited treatment modalities that were available that time. As medical treatments have improved the last decade, more and more "immature" infants survive. Histologic examinations of those infants that die of BPD reveal other characteristics than the lungs of infants dying of BPD that were described by Northway et al. Lungs of infants with BPD born after 28 weeks gestational age show merely fibrosis and inflammation. The lungs of more immature infants born at 24-28 weeks gestational age, suffering from BPD, show signs of an arrest in lung maturation with fewer and larger alveoli and less striking fibrosis and inflammation.^[40,41]

Lung maturation is influenced by multiple factors. Antenatal glucocorticoids induce structural maturation and induce the surfactant system.^[42,43] However, they also interfere with alveolarization.^[44,45] Chronic low-grade inflammation or infection is another factor in lung

maturation. Although infants born after with inflammation of fetal membranes and amniotic fluid, which is the case in about 50% of preterm deliveries, have a decreased incidence and severity of RDS, they have an increased incidence of BPD.^[41,46] Therefore the role of inflammation is assumed to be very important in the development of BPD. Chronic antenatal inflammation, probably leading to a decreased incidence of neonatal RDS, is considered to be a cause of BPD in the immature infant. Persistent inflammation after neonatal RDS is considered to be a cause of BPD in the more mature infant.

Prevention.

The best prevention of neonatal RDS would be the prevention of preterm birth.

Preventive strategies aiming at prevention of neonatal RDS after (inevitable) preterm birth, have been directed at acceleration of lung maturation and maturation of surfactant synthesis. Meta-analysis of antenatal glucocorticoid administration to the mother has shown that this is really effective in reducing the incidence and severity of neonatal RDS in preterm infants (over all odds ratio 0.5, 95% confidence interval 0.40-0.65.^[47] Glucocorticoids stimulate the production of surfactant proteins and phospholipids. They also stimulate lung maturation by enhanced maturation of cell differentiation, especially of type II cells and fibroblasts, by inhibition of DNA synthesis and thereby inhibition of cell synthesis, but consequently stimulation of cell differentiation. Glucocorticoids also cause a decrease in interstitial tissue in the alveolar septa, which makes the alveolar septa thinner which facilitates gas-exchange.^[48] Another important effect of glucocorticoids is the protection against oxidative damage. Recently an inhibitory effect of glucocorticoids on the development of pulmonary edema has been described.^[49] Glucocorticoids influence water reabsorption by decreasing pulmonary vascular resistance and by increasing aquaporin-1 water channel mRNA.^[48,50,51]

However, the use of antenatal glucocorticoids also has disadvantages. Although clinical trials of maternally administered corticosteroid show no evidence of increased disability on (short term) follow up, animal studies have shown that maternal corticosteroid administration delays myelination and reduces the growth of all fetal brain areas particularly the hippocampus. Corticosteroids may enhance hypoxic-ischaemic injury to the developing brain depending on timing and dosage.^[52] The other risks of especially multiple courses of antenatal glucocorticoids principally include restricted intrauterine growth, postnatal insulin resistance and changes to the hypothalamic-pituitary-adrenal axis.^[53] Antenatal glucocorticoid exposure may lead to adult cardiovascular and/or metabolic disease: an animal model suggests influence of glucocorticoids on the developing renin-angiotensin system.^[54]

Therefore other preventive strategies have been tried, such as antenatal thyroid releasing hormone administration.^[55] Thyroid hormone increases synthesis of surfactant phospholipids and accelerates structural development of the connective tissue matrix of the

lung.^[56-58] However, it also decreases surfactant protein A and B production and decreases lung antioxidant enzyme activity.^[58,59] Recent multicenter trials showed no additional effects of antenatal thyroid hormone administration to antenatal glucocorticoid administration or postnatal surfactant administration.^[55] Antenatal thyroid releasing hormone still could be considered in those situations where surfactant is not available.^[60]

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Chapter 3

The role of inflammation in neonatal RDS.

The role of inflammation in neonatal RDS.

Introduction.

Inflammation in general is the host's response to any injury or threatening damage, for example: trauma, infection, ischemia, hyperoxia. Inflammation within the lung of preterm infants can therefore be considered to be an appropriate defense mechanism against injury and infectious processes. However, persistence of polymorphonuclear leukocytes (PMNs) and macrophages in the lungs of preterm infants accompanied the development of bronchopulmonary dysplasia (BPD). In contrast, PMNs and macrophages disappeared if neonatal respiratory distress syndrome (RDS) resolved and no BPD developed. Therefore, concern has arisen about the appropriateness of the inflammatory reaction in preterm infants.

In this chapter first the normal systemic inflammatory reaction will be reviewed. The lung is an important site of host defense. In the lung capillaries more PMNs can be found than in the systemic circulation. The special characteristics of the inflammatory reaction in the lung will be separately discussed. The inflammatory reaction has to be regulated securely. It has an important function in the defense of the body against injury and infectious agents, but this reaction can be injurious to the own body if not stopped in time or attenuated sufficiently. Therefore complex pro- and anti-inflammatory processes regulate the inflammatory reaction. These mechanisms will be discussed afterwards. Neonates have an immature immune system when compared to adults. What we know about the regulation of the inflammatory reaction is mainly derived from adult humans or animal studies. Known differences between adults and neonates and especially preterm neonates will be described. As the subject of this chapter is the role of inflammation in the development of symptoms of RDS we proceed with the description of the relationship between inflammation and neonatal RDS. As the importance of the inflammatory reaction has been established very well in BPD and this was the basis for interest in the role of inflammation in neonatal RDS, this chapter will end with some short comments concerning the relationship of inflammation and BPD.

The normal systemic inflammatory reaction.

The normal inflammatory reaction is a response to any cellular injury that serves as a mechanism initiating the elimination of the noxious agent and damaged tissue. The reaction is marked by capillary dilatation, leukocyte infiltration and can result in redness, heat, pain, swelling and loss of function. The inflammatory reaction can be divided in an innate system and an adaptive system. As the initial reaction in neonatal RDS concerns the innate reaction, the rest of this review will be focused at this innate reaction.

Any tissue damage or infection can initiate the innate reaction. In response to injury or microorganisms, macrophages, monocytes, and PMNs are the first line defense cells. They bind to body foreign material, for example microorganisms, and kill them. PMNs therefore have to migrate from the blood stream to the injured tissue. In normal vessels, PMNs move rapidly. Under influence of selectins that are expressed on endothelial cells (E-selectin and P-selectin) and PMNs (L-selectin), PMNs roll over the endothelium. P-selectin is mobilized to the surface of endothelial cells in response to platelet-derived factors, thrombin, histamin or complement. Pro-inflammatory cytokines as tumor necrosis factor α (TNF- α) and interleukin-1 (IL-1) and IL-8 stimulate the expression of selectins on PMNs (figure 1).

Next, integrins that are stored in granules in the PMNs, are released to the surface of the PMNs. Together with integrins that are already present at the surface of PMNs, they are activated. Different chemotactic molecules, for example LTB₄, C5a, IL-8 and macrophage inflammatory proteins mediate release and activation of integrins. Activation and increased expression of integrins will cause sticking of the PMNs to the endothelium. After sticking to the endothelium, the PMNs adhere closely to the endothelium by binding of the activated integrins to intercellular adhesion molecules expressed on endothelial cells (ICAM-1, ICAM-2) and platelets (PECAM-1). The last step is diapedesis and transmigration of the PMNs from the vascular compartment to the site of inflammation, which is mainly mediated by binding of PECAM-1 to integrins (figure 1).^[1]

Figure 1. The various steps of the adhesion cascade. After endothelial activation by cytokines derived from inflammatory tissues, selectins are expressed on the endothelium. The interaction between selectins and their leukocyte ligands will start the rolling process. This will lead to leukocyte activation with increased expression of the integrins which will cause the sticking of the cells to the endothelium. In the last phase, transmigration is mediated mainly by binding of platelet-endothelial cell adhesion molecule-1 (PECAM-1) to integrins.

At the site of inflammation, the PMNs secrete direct bactericidal mediators, oxygen radicals, bioactive lipids, cytokines as well as proteases (for example elastase and β -glucuronidase).^[2] Elastase and β -glucuronidase are stored in the PMNs in the azurophilic granules. They can be released immediately after activation of the PMNs.^[3] Elastase is inactivated by α_1 -proteinase inhibitor especially in plasma, but β -glucuronidase remains in the circulation and can be measured and used as a marker for systemic activation of PMNs.

The special characteristics of the inflammatory reaction in the lung.

In normal healthy subjects only a few PMNs can be found in the alveoli. However, the lung is a major organ in host defense because it has a large surface area that is in contact with the external environment and because the lungs receive all venous blood from the body. The capillary bed of the lungs contains a large reservoir of leukocytes, mainly PMNs, which are in close contact to the endothelium, the so-called margined pool, which can be recruited easily if necessary.

The margined pool is a special characteristic of the lung capillaries. Concentrations of leukocytes are 40 to 65 times higher than concentrations found in large vessels.^[4,5] The formation of this pool is caused by longer transit times of PMNs than red blood cells in the narrow lung capillaries. The transit time of PMNs through the lung capillaries is median 26 seconds, whereas the transit time of red blood cells is 1.4-4.2 seconds. Because the diameter of leukocytes (diameter 10-12 μm) is larger than the diameter of erythrocytes (diameter 7 μm), they have to deform more to pass through the narrow lung capillaries than erythrocytes, which takes more time. The prolonged transit time seems helpful in host defense because it leaves more time to the cells to detect an inflammatory site. Adhesion molecules do not mediate the formation of the margined pool.

In response to lung injury (for example by positive pressure ventilation) or after bacterial invasion or endotoxin release, PMNs become also activated in the lung capillaries. Rolling, as the first step in adherence of the PMNs to the endothelium is not possible in the narrow lung capillaries as PMNs are deformed to an elongated and narrow form to make passage possible. $\text{TNF-}\alpha$, IL-1 and IL-8, produced by activated macrophages in the lungs, activate the PMNs. In response the PMNs become enlarged and more rigid, which further prolongs transit time and leads to retention in the lung capillaries. This process can take place without the complex interaction between integrins on the PMNs and cellular adhesion molecules on the endothelial cells as in systemic circulation. After sequestration of the PMNs, also platelets accumulate in the lung capillaries. Platelet-neutrophil interaction contributes to the activation of the PMNs. In this phase the interaction between L-selectin and integrins (CD11/CD18) on the PMNs, and cellular adhesion molecules on the endothelial cells, is necessary to keep the PMNs sequestered.^[4] Thereafter diapedesis through the endothelial cell lining of the capillaries takes place, which can be integrin dependent as well

as integrin independent. PMNs can migrate directly through discontinuities in the endothelial membrane (figure 2).^[6]

Figure 2.
Postulated sequence of events
in the response of PMNs to
inflammatory stimuli in the
lungs.

The next important step is emigration of the PMNs out of the capillaries into the alveoli if that is the site of inflammation. In the lungs this is a complex path that has not been unraveled completely. It seems to take place as well integrin dependent as integrin independent. It is a complex mechanism regulated by interactions of the PMNs with fibroblasts and the matrix. The fibroblasts form a track through the interstitium between the endothelium of the capillaries to the epithelium where they interact with the type II alveolar cells. In inflammation, activated PMNs migrate through disrupted junctions between endothelial cells and disrupt the endothelial-fibroblast contact. They then migrate along the disrupted fibroblast to the type II alveolar cell, pass into the intercellular space between type I and type II cells and arrive into the alveolar space (figure 3).

In the alveolar lumen, the activated PMNs release mainly elastase, which is stored in azurophilic granules in the PMNs. Elastase is the major granule component. In healthy individuals, α_1 -proteinase inhibitor inactivates elastase, but elastase is able to escape regulation at inflammatory sites.^[7] It is capable of degrading elastin, but it can also degrade some types of collagen and fibrinogen. In addition, elastase can activate and inactivate complement factors C3 and C5a, thereby regulating the inflammatory reaction. Elastase is therefore involved in the pathophysiology of acute lung injury.^[7]

Figure 3.
*PMN migration through the
alveolar-capillary wall into the
alveoli.*

Pro- and anti-inflammatory mechanisms.

As stated before, the inflammatory reaction is a defensive response that is directed against injury and infection. The innate immune system recognizes pathogen-associated patterns and reacts with the release of mediators that protect against injury or infection.^[8] However, the overzealous release of inflammatory mediators can cause excessive tissue injury and can be directed against the host itself. Ongoing activation of inflammation can result in multiple organ dysfunction and frequently death, which we know from the so-called Systemic Inflammatory Response Syndrome (SIRS).^[9] Hence, the inflammatory reaction has to be regulated appropriately.^[10] The inflammatory reaction can be considered as a coin with SIRS being the one side and Compensatory Anti-inflammatory Response Syndrome (CARS) being the other side (figure 4).^[9,11-13]

Numerous pro-inflammatory stimuli and cytokines can induce the SIRS side of the coin.^[14] Sepsis, trauma or extracorporeal bypass treatment may initially induce the first stage(s) of the systemic pro-inflammatory reaction and lead to extensive substance release.^[15] More and more aspects of the molecular basis of the systemic inflammatory response are explored. IL-1 and TNF- α are the first cytokines in the inflammatory response, immediately followed by interferon gamma (IFN- γ), IL-12, IL-18 and the granulocyte-macrophage colony-stimulating factor (GM-CSF).^[10]

Recently the great impact of this response and its products on the lung is addressed in many papers.^[7,16-19] The activated neutrophils in the systemic circulation release cytokines, proteases and oxidants.^[20] Nearly every pulmonary cell simultaneously reacts on IL-1 and TNF- α with expression of endothelial adhesion molecules, increased endothelial permeability, increased blood flow through smooth muscle relaxation via the nitric oxide pathway and the production of IL-8 by endothelium, epithelial cells and fibroblasts.^[17] Besides IL-1 and TNF- α , other mediators as complement factors C3a, C5a, platelet-activating

factor, metabolites of arachidonic acid, endotoxins, and the coagulation cascade in acute lung injury, are pro-inflammatory. Even therapeutic interventions like artificial ventilation have shown to be pro-inflammatory and play an important role in the acute phase of lung injury.^[16,21]

Figure 4.
The relationship between SIRS, CARS and mixed antagonistic response syndrome (MARS), and the factors that influence the individual patient.

An important role in the upregulation of pro-inflammatory cytokines in the acute phase is reserved for the Nuclear Factor κ B/ Inhibitor of κ B Kinase (NF- κ B/IKK) signal transduction pathway.^[22] Two different pathways are presently known. The first one is the canonical pathway which liberates NF- κ B heterodimers that are translocated into the nucleus where they induce genetic upregulation of cytokines and growth factors leading to activation of innate immunity and inflammation response. The second pathway is the IKK- α specific pathway that is required for adaptation of the B cell mediated immunity.^[23]

Also the macrophage migrating inhibiting factor is directly pro-inflammatory. It promotes the expression of TNF- α , IL-1 β , IL-2, IL-6, IL-8, IFN- γ , nitric oxide release, matrix

metalloproteinase-2 expression and induction of the cyclooxygenase-2 pathway.^[24] At the same time macrophage migrating inhibiting factor counteracts glucocorticoid-induced inhibition of pro-inflammatory cytokine secretion by macrophages (TNF- α , IL-1 β , IL-6, IL-8) and T cells (IL-2 and IFN- γ).

Phospholipase A₂ is an important enzyme for the formation of several pro-inflammatory mediators, for instance arachidonic acid metabolites plus platelet activating factor and by doing so it is considered a critical mediator of the inflammatory reaction.^[16] Neutrophils, platelets and possibly alveolar macrophages are the source of phospholipase A₂ in bronchoalveolar lavage fluid.

At the cellular level, complex control mechanisms normally regulate the on-off-switch of inflammatory cells. Interconnected intracellular signal transduction pathways regulate cellular responses by activating effector proteins and by modifying protein expression and transcription. At the molecular level the mitogen-activated protein kinase superfamily, with its important three members: extra-cellular signal-regulated kinase, p38 and c-Jun N-terminal kinase, is one of the major mechanisms used by human cells to transduce extracellular signals into a cellular response.^[13] This leads to the speculation that analysis of the activation status of mitogen-activated protein kinase cascades can identify patients at high risk for SIRS and multi organ dysfunction syndrome. Especially p38 is assumed to mediate expression of vascular endothelial growth factor (VEGF) on macrophages by lipopolysaccharides. VEGF is a protein that plays an important pro-inflammatory role and it enhances vascular permeability. P38 mitogen-activated protein kinase inhibitors can inhibit the induction of VEGF and the production of the VEGF protein itself can be inhibited by dexamethasone. These observations may give impulses to the development of new and early ways of (antenatal) treatment of inflammatory reactions in the future.^[25]

The CARS side of the coin is a complex process because counteracting or down regulating the inflammatory reaction is a delicate process, where cytokines, some chemokines and different growth factors play crucial roles. Many pro-inflammatory mediators also have anti-inflammatory properties; sometimes respective of the body compartment they are in.^[9,14,26] Where IL-1 shows only moderate anti-inflammatory properties, the IL-1-receptor antagonist is much more potent in this perspective. IL-4 and granulocyte colony stimulating factor (G-CSF) both show more anti- than pro-inflammatory effects.^[27] IL-10 shows only anti-inflammatory activities. It is synthesized in monocytes, macrophages, epithelial and T-plus B-cells in response to all kinds of bacterial, viral and fungal invaders as well as several cytokines (TNF- α , IL-1, IL-6, IL-12).^[28,29] The effect of IL-10 is many-sided; it suppresses the synthesis of IL-1 α , IL-1 β , TNF- α , IL-6, IL-8, IL-12, IL-18, GM-CSF, GC-CSF, macrophage inflammatory protein-1 α plus its own production (see figure 5).^[29] An important action of IL-10 is also the inhibition of translation of NF- κ B.^[30,31] IL-10 can also induce apoptosis of neutrophils and T-lymphocytes.^[32] However IL-10 induced increase of the expression of the Bcl-2 protein is reported leading to preservation of B-cells.^[33]

Figure 5. Interaction of IL-10 with other cytokines.

Differences between levels of IL-10 and IL-10 receptor antagonists in plasma and bronchoalveolar lavage fluid were reported in adult patients with acute respiratory distress syndrome pointing to different compartments during the inflammation process. Survivors of adult respiratory distress syndrome showed significant higher IL-10 and IL-10 receptor antagonist levels compared to non-survivors.^[34] In primates, administration of IL-10 markedly reduced the inflammatory reaction and it also reduced the hypotension associated with endotoxin administration without influence on coagulation or fibrinolytic systems.^[35] However, Pajkrt et al. reported that IL-10 administration reduced both activation and inhibition of the fibrinolytic system.^[36] More trials are needed especially in preterm infants before treatment with exogenous IL-10 can become a standard treatment.

Newer insights in the cellular protection mechanisms against harmful effects of the pro-inflammatory response point to the Heat Shock Proteins and the so-called Heat Shock Response. Heat Shock Proteins act as chaperone proteins to protect vital protein structures and functions.^[37] Besides its own palette of action the Heat Shock Response interacts with the NF- κ B pathway both by increased inhibition of IKK and increased phosphatase activity, resulting in a net decrease of phosphorylated I κ B α by which the activity of NF- κ B is inhibited.^[38]

The balance between pro- and anti-inflammation is delicate and subtle and much more research is needed to provide better insight in how, when and who. The report that the Bax-gene plays a role in neutrophil apoptosis and that cytokines can induce Bax deficiency leading to more effector cells in inflammation may induce research for increased sensibility for SIRS in certain subgroups in the population.^[39]

Differences between inflammatory properties of neonates and adults.

Much and specific knowledge of PMN function and inflammatory reaction is available for adults, while pediatric literature merely focuses on differences in host defense against pathogens due to the high risk for severe bacterial and fungal infections especially in preterm infants. SIRS and CARS and their clinical features are mainly described in older infants and adults, incidentally in neonates but not in preterm infants.^[40-42]

As stated before, data concerning the neonatal inflammatory response are available mainly from an infectious point of view. Concerning the innate immune response, newborns have a relative neutropenia compared to adults. The neutrophil storage pool is decreased compared to adults and neutrophil production is diminished.^[43] Although newborns have high levels of G-CSF, which are even higher in preterm infants than in term infants, a relative G-CSF deficiency may be present during infection.^[44] Even with high levels of G-CSF the newborn has a limited capacity to accelerate his neutrophil production during infection and it may be presumed that this is also the case in a non-infectious inflammatory situation.^[45] In utero and at birth the lung is considered a relative easy porte-d'entree for different invaders due to the limited pulmonary margined pool of PMNs caused by the almost complete block of pulmonary blood flow in utero. Additionally, from experiments with pre-term rabbits we know that the number of alveolar macrophages is very limited.^[46]

Besides the limited numbers of PMNs, also the decreased deformability of the PMNs from a newborn is an important factor why less neonatal PMNs can pass the endothelium.^[47] Cytoskeletal components as actin and tubulin seem to be responsible for the differences.^[47-53]

Neutrophils from neonates show decreased adherence to surfaces, even under stimulated conditions. Compared to adult neutrophils the adhesion of neutrophils from neonates is only 40-45%.^[54-60] The reasons are decreased L-selectin and a failure of upregulation of β_2 -integrins (Mac-1) after exposure to chemotactic agents.

Present data do not point to a decreased number of Fc receptors at the surface of the PMNs in the healthy term infant. However the preterm infant has a reduced Fc receptor III capacity at the surface of his PMNs leading to a decrease in opsonisation. This all results in less PMNs reaching the site of infection and presumably the site of inflammation compared to adults.^[56]

The ability of PMNs of neonates to release lysosomal enzymes as lysozyme and proteases as β -glucuronidase are at an adult level in general.^[61,62] Superoxide anion, which is

important for intracellular killing, is produced in the same amount by PMNs of term newborns compared to adult PMNs.^[60] However, the production of superoxide anion by PMNs of cord blood of preterm neonates seems to be reduced.^[62,63] Decreased amounts of the hydroxyl radical are demonstrated in the newborn infant.^[64] Data on bacterial killing are somewhat confusing because the situation of (serious) infection versus healthy status seems to have impact on the results of the tests.^[54]

Data on systemic inflammatory response and the molecular regulation of the inflammatory reaction are described mainly in adults. For the future it is necessary that data concerning term and preterm infants will be gathered. The reason to do this is striking because a maternal systemic inflammatory reaction seems to have great impact on morbidity and mortality of the fetus.^[65] In 74 obstetric patients with SIRS admitted to the Intensive Care Unit, five spontaneous abortions and eight perinatal deaths were recorded. No data of inflammatory parameters in the children were available.

Role of inflammation in the development of symptoms of neonatal RDS.

Any activation of the inflammatory reaction means starts with the extensive release of pro-inflammatory cytokines, (TNF- α , IL-6, IL-1), chemokines (IL-8, MIP-2), lipid mediators (LTB₄, platelet activating factor, prostaglandins) from neutrophils, monocytes, platelets, airway epithelial cells and endothelial cells and to complement activation. This leads to activation of the endothelial cells and movement of macrophages and PMNs from the vessels to the interstitial space and the alveoli as described above.^[66]

The immediate effect of the inflammatory reaction is increased vascular permeability leading to plasma protein leakage to the interstitium and the alveolar spaces.^[1,67,68] The increased permeability is dependent of gestational age: with decreasing gestational age, protein leakage increased in preterm lambs.^[67] The result of protein and fluid leakage to the alveoli is inhibition of surfactant function by interference with formation of the surfactant monolayer.^[69,70] Whenever there is impaired surfactant function, respiratory distress can develop, but in preterm infants with already existing surfactant deficiency, this effect is more pronounced.

Not only local activation of the inflammatory reaction influences lung vascular permeability. Lung vascular permeability was dependent of PMN accumulation in the alveoli but also of PMN disappearance from the circulation in preterm lambs.^[71] In infants this phenomenon could also be demonstrated. The loss of circulating PMNs 2 hours after delivery was associated with more severe respiratory distress during the first postnatal week.^[72] Whether this effect was due to accumulation of PMNs in the lung or the result of systemic activation of inflammation is unknown. In patients with adult respiratory distress syndrome a systemic increase in inflammatory mediators preceded the clinical or radiologic signs of respiratory failure and adult respiratory distress syndrome.^[73]

PMNs play a key role in the defense of the lung after being attracted to the site of injury. After migration to the alveolar space, PMNs release oxygen radicals, neutral proteases and lysosomal enzymes. Oxygen radicals are considered major bactericidal agents and in the PMNs, IL-1 and TNF- α enhance phagocytosis of bacteria and foreign material (for example cell rests after lung injury due to mechanical ventilation). Proteases can degrade the subendothelial matrix, thereby damaging the lung structure. The main protease, elastase, if not inactivated by α_1 -proteinase inhibitor, is very important in the pathophysiology of acute lung injury.^[7] Elastase has direct pro-inflammatory activity by influencing neutrophil chemotaxis and adhesion and by inducing the release of pro-inflammatory cytokines. Elastase may as well degrade cell junction proteins, thereby increasing alveolar capillary permeability. Elastase also degrades elastin, some collagens, fibrin and fibronectin. Elastin and collagen are components of the main framework (alveolar septa) of the lungs. Elastin and collagen degradation by elastase therefore has a destroying effect on the lung architecture.^[74] Fibronectin is an opsonin that is stored in fibroblasts, macrophages and epithelial cells and that also is present in plasma and interstitial fluid.^[75,76] After release in response to injury, it has multiple functions. It promotes clearance of fibrin, platelets, immune complexes and cell rests. This means it has “healing” properties. However, fibronectin also functions as an attractant for phagocytes, as well as PMNs and fibronectin can also directly destroy healthy tissue, thereby contributing to an increased inflammatory reaction and tissue fibrosis.^[75,77] The interaction of fibronectin with PMNs is therefore complex: fibronectin attracts PMNs, which release elastase that degrades fibronectin, which has regenerating as well as damaging properties. In addition, PMNs show cytotoxic activity towards lung interstitium cells through the peroxidation of membrane lipids, a process that is mediated by oxygen radicals.^[2]

Comments concerning relationship inflammation and BPD.

To downregulate the inflammatory reaction, reversing the inflammatory reaction into a healing process, feedback mechanisms between inflammatory cells and the mediators are very important. Anti-inflammatory cytokines (IL-4 and IL-10, cytokine receptor antagonists and other suppressive mediators (prostaglandin E2) are all released by the inflammatory cells (macrophages, PMNs, lymphocytes) present at the site of injury, hence controlling the inflammatory reaction. However, if the inflammatory reaction is not down regulated, because of ongoing injury, for example prolonged barotrauma or oxygen toxicity from mechanical ventilation or infectious disease, the inflammatory reaction may persist, or may be amplified. This will lead to the persistence of inflammatory cells in the alveoli, proliferation of fibroblasts, altered collagen synthesis and eventually matrix distortion. The result will be tissue injury, fibrosis and possibly impairment of the normal development of the lung.

Already at 4 days of postnatal age, infants who develop BPD have higher levels of PMNs and higher levels of alveolar macrophages in airway samples than infants recovering

from RDS. The inflammatory cells persist in BPD infants, whereas they disappear in recovering infants.^[78-82] The influx and persistence of inflammatory cells in airway samples of infants with developing BPD is accompanied and is even preceded by high levels of pro-inflammatory cytokines. Munshi et al. found that IL-8 on day 1 and day 3 and IL-6 on day 3 and day 5 was elevated in infants developing BPD. The PMNs were higher in infants developing BPD after day 5.^[82] Tullus et al. found higher levels of IL-1, IL-6, IL-8 and IL-1 receptor antagonists in infants developing BPD.^[83] Buron et al found that the level of ICAM-1 in alveolar fluid after day 2 was higher in infants developing BPD than in infants recovering from RDS.^[84,85]

Groneck et al. related the presence of pro-inflammatory cytokines and other chemo-attractants to the increased microvascular permeability that can be found in infants developing BPD. They found increased chemotactic activity, higher number of PMNs and increased levels of IL-8, C5a and LTB4 in airway samples of infants with developing BPD compared to infants without BPD. This inflammatory reaction is associated with increased pulmonary epithelial leak, which is caused by the influence of cytokines and other inflammatory mediators on the structure of glycosaminoglycans.^[79] The altered structure of the glycosaminoglycans alters the surface charge of the endothelial membrane, which enables small proteins (for example albumin) to leak from the plasma through the interstitium to the alveoli.^[86,87]

Not only activators of inflammation, but also end products of activated inflammatory cells can be found in airway samples of infants developing BPD. Elastase and an imbalance between elastase and its inhibitor α_1 -proteinase inhibitor can be demonstrated in tracheo-bronchial fluids of infants developing BPD.^[78,88] Degradation of elastic fibers by free elastase, which leads to impaired alveolar septation, contributes to acute and chronic lung injury.^[87]

Nowadays, the lungs of more immature infants born at 24-28 weeks gestational age, suffering from BPD, show histologic signs of an arrest in lung maturation with fewer and larger alveoli and an arrest in vascular development. Signs of inflammation and fibrosis are less striking than in the BPD of infants born after 28-32 weeks gestational age.^[90] However inflammation still plays an important role. Histologic chorioamnionitis is associated with a decrease in the incidence of RDS but an increase in BPD. Elevated levels of cytokines (IL-1 β , TNF- α , IL-6, IL-8) in amniotic fluid are associated with an increased risk of BPD.^[91,92]

In summary it is known now that the inflammatory reaction in the lungs by retention of PMNs is not the same as the systemic inflammatory reaction. Neonates and especially preterms with their immature PMNs, differ in their response to inflammatory stimuli from adults. Inflammation locally in the lungs and RDS itself play an important role in the development of BPD. Whether an early activation of the inflammatory reaction, i.e. immediately after delivery, is related to the development of neonatal RDS is the aim of the studies we performed.

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Chapter 4

Early activation of inflammation and clotting in the preterm lamb with neonatal RDS: comparison of Conventional Ventilation and High Frequency Oscillatory Ventilation.

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Abstract.

In neonatal respiratory distress syndrome activation of inflammation and clotting is demonstrated. High Frequency Oscillatory Ventilation (HFOV) is considered to be less damaging to the human preterm lung, resulting in less activation of inflammation and clotting compared to Conventional Ventilation (CV).

To assess the sequence of events of activation of inflammation and clotting and to compare the impact of HFOV to CV, we ventilated preterm lambs delivered by cesarean section at 132 days gestational age (term 145 days) for 8 hours by CV (n=10) or HFOV (n=11). 15 Minutes after birth and at 2 hour intervals thereafter blood samples, from umbilical catheters, were analyzed for AP50 (complement activation), number of polymorphonuclear leukocytes, β -glucuronidase, platelet function, activated partial thromboplastin time, thrombin time and thrombin inhibition, and bronchoalveolar lavage fluid was analyzed for elastase, thrombin and protein.

We found complement activation, low number of polymorphonuclear leukocytes and high levels of β -glucuronidase already at 15 minutes after birth. Within 2 to 4 hours after birth platelet function deteriorated, activated partial thromboplastin time prolonged and thrombin inhibition decreased. Activation of inflammation and clotting in the lungs was demonstrated by increased levels of elastase and thrombin in and bronchoalveolar lavage fluid. In the HFOV group, AP50 remained significantly higher than in the CV group, reflecting less complement activation, and platelet function analysis remained significantly lower, reflecting better platelet function.

We conclude that systemic activation of inflammation can be found in the ventilated preterm lamb with respiratory distress syndrome within 15 minutes after birth. Afterwards, or due to activation of inflammation, clotting is activated. HFOV possibly attenuates activation of inflammation.

Introduction.

Neonatal Respiratory Distress Syndrome (RDS) occurs in preterm infants, due to structural immaturity of the lungs and immaturity of the pulmonary surfactant metabolism, resulting in surfactant deficiency and surfactant dysfunction. Apart from these factors there is increasing evidence that inflammation and clotting contribute to the progression of this disease.

Already in 1983, Merritt et al. described that 3 days after birth an enhanced inflammatory response could be found in bronchoalveolar lavage fluid (BALF) of infants developing bronchopulmonary dysplasia after RDS.^[1] Other investigators confirmed this observation. Murch et al. found in the lungs of infants who died because of acute RDS, excessive polymorphonuclear leukocytes (PMNs) and alveolar macrophages.^[2] Other authors found PMNs, alveolar macrophages and pro-inflammatory cytokines in BALF, reflecting local activation of inflammation in the lungs of preterm infants with RDS, prone to development of bronchopulmonary dysplasia.^[1,3,4-7,8]

However, as is known for ARDS and shock in adults, not only local activation of inflammation could be expected, but also systemic activation of inflammation.^[9] Recently Brus et al. reported that in RDS and chronic lung disease in preterm infants, systemic activation of inflammation could be found also.^[10,11]

Clotting also plays an important role in the pathogenesis of RDS. As is known from histopathologic studies, fibrin is a major part of hyaline membranes and therefore hyaline membranes can be regarded as locally produced clots in RDS.^[12,13] Schmidt described that intravascular thrombin-antithrombin III complex formation was related to disease severity in RDS.^[14] A similar observation was described by Brus et al. who investigated the systemic activation of clotting and fibrinolysis. A correlation was found between activation of clotting and the severity of protein leakage in the lungs of preterm rabbits as well as between the activation of clotting and the clinical severity of RDS in preterm infants.^[15,16] These studies strongly suggest that apart from inflammation, local as well as systemic activation of clotting is involved in the pathogenesis of RDS.

In the light of the complex interaction between the inflammatory reaction and clotting, this finding is not surprising. However, the sequence of events in terms of local and systemic activation of inflammation and clotting remains to be elucidated.

It is suggested that both inflammation and clotting can be activated as the result of mechanical ventilation on the preterm lung.^[17] Since preterm lambs could not survive without mechanical ventilation, we decided to introduce high frequency oscillatory ventilation (HFOV) in one of the groups of lambs, being a mode of ventilation that is considered to be less damaging to the human preterm lung.^[18,19]

Hence the aim of the study was to investigate the sequence of events in local as well as systemic activation of inflammation and clotting in the preterm lamb with RDS and to compare the impact of different ventilatory modes (conventional ventilation [CV] vs. HFOV).

Methods and materials.

The experiments were performed in the central animal laboratory of the University of Groningen, under approved animal care protocols of the University of Groningen with concern for animal welfare. We studied 21 preterm lambs of 132 days gestational age (term 145 days). We chose lambs of 132 days because it is known that at this gestational age, lambs suffer from RDS.^[20] We saw in a pilot study that lambs that were not ventilated, died with the clinical picture of RDS: tachypnea, dyspnea, grunting, cyanosis, respiratory acidosis, high oxygen need and ultimately respiratory failure.

Treatment of the animals.

The ewes were sedated with Sevoflurane inhalation anesthesia and a cesarean section through a lateral incision was performed under local anesthesia with subcutaneous prilocaine. Ewes were not selected for twin pregnancy. Before starting each single experiment we randomized the first-born lamb to CV or HFOV by coin method. If there was a twin pregnancy, the second lamb underwent the same procedure as the first-born lamb, but was assigned to the alternate ventilatory mode.

The lambs were delivered and, before clamping and cutting of the umbilical cord, they were intubated orotracheally with a cuffed 5.5 mm ID tube and the airways were suctioned once. After delivery the lambs were dried and weighed and mechanically ventilated immediately with a time-cycled pressure-limited infant ventilator (Babylog 8000, Dräger, Lübeck, Germany) for CV or a High Frequency Oscillatory Ventilator (Sensor Medics 3100, Yorba Linda, California, USA) for HFOV.

Initial ventilator settings of CV were: frequency 60/min, inspiratory time equal to expiratory time at 0.5 sec., gas flow 8 l/min. Peak inspiratory pressure was initially set at 20 cm H₂O and was adjusted immediately after the start of ventilation to achieve a tidal volume of 5-8 ml/kg and an expiratory minute volume of about 250 ml/kg/min. Positive end-expiratory pressure was set at 4 cm H₂O. Fractional concentration of inspired O₂ (FiO₂) was set at 0.4 and adjusted to maintain transcutaneous O₂ saturation between 92 and 95% (measured with Pulseoximeter Nellcor, Hayward, California, USA) and PaO₂ between 6.0 and 8.0 kPa. During the study peak inspiratory pressure was adjusted to achieve PaCO₂ values of 5.5 to 7.0 kPa provided that pH was > 7.25.

Initial ventilator settings of HFOV were: frequency 10 Hz, inspiratory time 0.33, gas flow 20 l/min. Mean airway pressure was initially set at 14 cm H₂O. Fractional concentration of inspired O₂ was set at 0.4 and after stabilization, we searched for optimal distending pressure, aiming at transcutaneous O₂ saturation between 92 and 95% and PaO₂ between 6.0 and 8.0 kPa with the lowest possible FiO₂ preferably below 0.3. Amplitude was initially adjusted according

to oscillatory movements of the chest wall and afterwards adjusted to achieve PaCO₂ values of 5.5 to 7.0 kPa provided that pH was > 7.25.

In all lambs, an arterial catheter was advanced in the descending aorta through an umbilical artery and was used for blood sampling and measurement of blood pressure. Patency was maintained by continuous infusion of a saline solution 0.9% at a rate of 2.0 ml/h. During the study blood pressure was maintained in the normal range (mean 40 mmHg). In case of hypotension, a 10 ml/kg bolus of saline solution 0.9% was administered intravenously. A venous catheter was advanced in the vena cava inferior through the umbilical vein and was used for the administration of fluids (3 ml/kg/h), glucose (5 mg/kg/min) and sodium pentobarbital. Serum glucose levels were measured two-hourly and were kept in the normal range (4-9 mmol/l) by adjusting the amount of glucose that was infused. Body temperature was measured continuously by a rectal thermometer. Body temperature was kept at 38°C by using preheated mattress and blankets. Sodium pentobarbital (Nembutal®) was given for sedation, initially 15 mg/kg/h, dosage was adjusted according to activity, blood pressure and heart rate of the lamb.

Blood sampling.

Blood samples of 0.4 ml were obtained hourly for bloodgas analysis in heparinized syringes. Blood samples of 5.25 ml were obtained immediately after inserting catheters (15 minutes after birth) and 2,4,6 and 8 hours after initiating ventilation. Five ml of this blood was anticoagulated with citrate 0.31% immediately and placed on ice. It was used to determine AP50, β -glucuronidase, platelet function, activated partial thromboplastin time (aPTT), thrombin time, thrombin inhibition and urea. The other 0.25 ml blood was anticoagulated with EDTA 0.01 M and was used to determine total leukocyte count, and to make blood smears for differential cell counts, especially to determine the number of PMNs.

Bronchoalveolar lavage.

Immediately after intubation, the airways of the lambs were suctioned. 2,4,6,8 hours after intubation, bronchoalveolar lavage was performed by injecting 1 ml/kg saline solution 0.9% intratracheally with immediate aspiration, this was repeated three times. To exclude effects of bronchoalveolar lavage on activation of inflammation and coagulation, we alternated bronchoalveolar lavage with dry suctioning in successive lambs. In aspirated material (from now on it is called BALF) elastase, thrombin and protein concentrations, corrected for urea, were determined. Between the lambs in whom bronchoalveolar lavage was performed and lambs in whom dry suctioning was performed, no differences in either ventilatory parameters, nor laboratory results were found, so the results of BALF of these lambs are combined for further analysis.

After 8 hours the study was ended, the lamb was euthanised with an overdose sodium pentobarbital, and a thoracotomy was performed to remove the lungs.

Processing of the samples.

The heparinized blood was used for blood gas analysis to assess PaO₂, PaCO₂, and pH, using an ABL 330 blood gas analyzer (Radiometer Co, Copenhagen, Denmark) and to assess hemoglobin and SaO₂ using an OSM-3 analyzer (Radiometer Co, Copenhagen, Denmark).

0.8 ml of the citrated blood was used directly to determine platelet function, using the Platelet Function Analyzer (DADE International Inc., Miami, USA). 3.7 ml of the blood was centrifuged at 1100 rpm for 10 minutes and the plasma was stored at –80°C until AP50, aPTT, thrombin time, thrombin inhibition and urea were determined.

The BALF and lung aspirate samples were centrifuged at 1100 rpm and the supernatant was stored at –80°C until further processing took place.

Assays.

AP50 titer (the alternative pathway hemolytic activity assay as a measure of activation of alternative route of complement system) is the plasma dilution that causes 50% lysis of non-sensitized rabbit erythrocytes in a standard suspension after prevention of activation of the classical route with EGTA and Mg²⁺.^[21] A reduced value indicates more complement consumption due to activation.

Determination of total leukocyte count from EDTA blood was performed microscopically using a chamber after a gentian violet staining. To determine differential cell counts, Giemsa stained smears were used. Total number of PMNs was calculated from these two measurements.

β-glucuronidase (released from PMNs after activation) was measured according to Baehner.^[22] For this purpose 25 μl lamb plasma was incubated with 25 μl p-nitrophenyl β-D-glucopyranosid uric acid solution for two hours at 37°C. The conversion of the substrate by β-glucuronidase was determined at 405 nm using a microplate reader (Microplate reader 3550 UV, Biorad, Richmond CA, USA). After determination it was corrected for the number of PMNs. Specificity of substrate used in the assay was tested by adding a cocktail of inhibitors (C1 esterase inhibitor, hirudine, trypsin and aprotinin), which inhibit complement, thrombin, trypsin, chymotrypsin, plasmin, kallikrein and activated protein C, to the test substance. We found no reduction of β-glucuronidase activity. So we concluded that the specificity of the substrate was good.

Platelet function analysis was carried out using the Platelet Function Analyzer, using PFA-100 collagen/ADP testcartridges (DADE International Inc., Miami, USA). This instrument reproduces the exposure to an injured vessel. In the Platelet Function Analyzer, platelets are

maximally stimulated to form a clot. Increased value of platelet function analysis is a sign of increased clotting because it takes longer to form a clot when the number of platelets is diminished or when the present platelets are “exhausted”, due to activated clotting *in vivo*. No references are known for the use of Platelet Function Analyzer in the analysis of the function of sheep platelets, but the reliability of the Platelet Function Analyzer has been described by Heilmann EJ et al.^[23]

The aPTT (a screening test for abnormalities in the intrinsic clotting system, especially factors VIII, IX, XI, XII, X and II, and prekallikrein, high molecular weight kininogen and fibrinogen) was measured by adding 100 µl aPTT reagent (Sigma Diagnostics, St. Louis, USA) and 100 µl calciumchloride to lamb plasma and recording clotting time.

Thrombin time, a screening test for the common pathway of the clotting system and especially measuring polymerization of fibrinogen to fibrin was measured by adding 100 µl thrombin time reagent (Sigma Diagnostics, St. Louis, USA) to lamb plasma and recording clotting time.

Thrombin inhibition (a method to determine the amount of mainly antithrombin III in plasma) was measured by adding excess thrombin stock (1.5 IU/ml, Biopool, Ventura, California, USA) to lamb plasma and measuring the remaining thrombin in the microplate reader at 405 nm. A higher value indicates more remaining thrombin, reflecting a lower antithrombin III concentration in the tested plasma.

Urea was determined according to Marsh et al.^[24]

Elastase in BALF (a measurement of local activation of PMNs in the lung) was measured by incubating BALF with elastase substrate (Omnilabo, Breda, The Netherlands) for three hours at 37°C. Afterwards the recorded optical density value at 405 nm using the microplate reader was compared to a standard porcine pancreatic elastase. Specificity of substrate used in the assay was tested by adding a cocktail of inhibitors (C1 esterase inhibitor, hirudine, trypsin and aprotinine), which inhibit complement, thrombin, trypsin, chymotrypsin, plasmin, kallikrein and activated protein C, to the test substance. We found no reduction of elastase activity. So we considered the specificity of the substrate as good.

Thrombin in BALF (a measurement of local activation of the clotting system in the lung) was measured after adding a thrombin specific substrate (Chromogenix S2238, Mölndal, Sweden). A higher optical density value at 405 nm using the microplate reader indicates a higher level of thrombin.

Protein in BALF (a measurement of capillary leakage due to local injury in the lung) was measured according to Lowry et al.^[25] A higher optical density value at 655 nm using the microplate reader indicates a higher protein level. To correct for dilution, values of elastase, thrombin and protein are corrected for the measured urea in BALF.

Data and statistical analysis.

Ventilatory data are presented as mean and standard deviation (SD). Laboratory data are presented as mean and standard error of the mean (SEM). For statistical evaluation Wilcoxon signed-rank test was used to determine changes between the first sampling moment and successive moments. Student's t-test was used to determine differences between groups. Data were considered significant when $p < 0.05$.

Results.

We studied 21 preterm lambs of 132 days gestational age. It was possible to ventilate preterm lambs of 132 days gestational age without surfactant, while maintaining adequate blood gas values. Ten lambs were ventilated with CV, 11 lambs were ventilated with HFOV.

Ventilatory and oxygenation parameters are shown in table 1 and table 2. Used FiO_2 , mean airway pressure, PaO_2 , $PaCO_2$, oxygenation index and alveolar-arterial partial O_2 tension difference did not differ significantly between the two groups.

CV (n=10)	t = 15 min	t = 2 hours	t = 4 hours	t = 6 hours	t = 8 hours	
Peak inspiratory pressure	28,4 ± 5,5	27,9 ± 5,1	28,2 ± 6,1	28,1 ± 7,0	27,5 ± 7,3	cm H ₂ O
Positive end-expiratory pressure	4,1	4,1	4,1	4,1	4,1	cm H ₂ O
FiO_2	0,71 ± 0,18	0,45 ± 0,20	0,40 ± 0,22	0,41 ± 0,25	0,38 ± 0,22	
Mean airway pressure	14,0 ± 2,0	14,8 ± 3,0	14,3 ± 2,1	14,2 ± 2,3	14,3 ± 2,8	cm H ₂ O
PaO_2	22,8 ± 16,9	8,1 ± 3,7	7,9 ± 2,9	9,4 ± 6,2	7,6 ± 2,1	kPa
$PaCO_2$	9,0 ± 2,3	6,0 ± 1,6	5,9 ± 1,8	6,4 ± 1,8	7,7 ± 2,0	kPa
OI *	11,6 ± 10,9	13,3 ± 9,1	11,2 ± 7,4	9,5 ± 6,1	10,2 ± 5,9	mm Hg
AaDO ₂ **	254 ± 178	212 ± 146	195 ± 147	171 ± 144	154 ± 143	mm Hg

Table 1. Ventilatory characteristics and blood gas analysis values for conventionally ventilated lambs at the moments that blood samples for measurement of study parameters were taken. Data are presented as mean ± SD.

$$* \text{OI} = \text{oxygenation index} = \frac{\text{Mean airway pressure} \times \text{fractional concentration of inspired } O_2}{PaO_2}$$

$$** \text{AaDO}_2 = \text{alveolar-arterial partial } O_2 \text{ tension difference} \\ = \text{fractional concentration of inspired } O_2 (P_{atm} - P_{H_2O}) - PaO_2 - PaCO_2$$

HFOV (n=11)	t=15 min	t= 2 hours	t= 4 hours	t= 6 hours	t= 8 hours	
Mean airway pressure	17,5 ± 4,1	13,9 ± 2,5	13,9 ± 3,3	12,9 ± 4,8	13,8 ± 3,7	cm H ₂ O
FiO ₂	0,60 ± 0,15	0,36 ± 0,15	0,29 ± 0,05	0,30 ± 0,06	0,30 ± 0,09	
PaO ₂	34,1 ± 16,7	8,8 ± 4,2	8,8 ± 5,0	8,4 ± 2,4	7,3 ± 1,9	kPa
PaCO ₂	5,9 ± 2,5	7,6 ± 7,4	5,7 ± 1,3	5,4 ± 1,2	6,3 ± 1,1	kPa
OI *	5,5 ± 3,7	9,3 ± 5,8	7,7 ± 3,6	7,3 ± 3,2	8,1 ± 4,0	mm Hg
AaDO ₂ **	148 ± 74	137 ± 70	100 ± 44	111 ± 40	130 ± 60	mm Hg

Table 2. Ventilatory characteristics and blood gas analysis values for high frequency ventilated lambs at the moments that blood samples for measurement of study parameters were taken. Data are presented as mean ± SD.

$$* \text{OI} = \text{oxygenation index} = \frac{\text{Mean airway pressure} \times \text{fractional concentration of inspired O}_2}{\text{PaO}_2}$$

$$** \text{AaDO}_2 = \text{alveolar-arterial partial O}_2 \text{ tension difference} \\ = \text{fractional concentration of inspired O}_2 (P_{\text{atm}} - P_{\text{H}_2\text{O}}) - \text{PaO}_2 - \text{PaCO}_2$$

In the CV group 3 lambs received saline (10, 20 and 30 ml/kg), in the HFOV group 2 lambs received saline (both 10 ml/kg). The mean (standard deviation) total dose of Nembutal in the CV group was 20.3 (11.8) mg/kg (total dose during the ventilation period), the mean (standard deviation) total dose of Nembutal in the HFOV group was 30.2 (13.9) mg/kg. Those dosages were not statistically significant different (Student t-test, p=0.1).

Hemoglobin levels remained stable in both groups during the day. Between the groups were no statistically significant differences (CV: mean hemoglobin 14.1 g/dl, SD 2.1, HFOV mean 13.3 g/dl, SD 2.4).

The figures 1a-1f show laboratory data of blood analysis including AP50, total number of leukocytes, number of PMNs, β-glucuronidase (corrected for number of PMNs), platelet function analysis and aPTT in plasma.

We detected the following changes in laboratory results concerning activation of inflammation and clotting in the preterm lambs with RDS.

AP50 (figure 1a) is already low within 15 minutes after birth (maternal sheep values of AP50 are about 200 IU/ml). A significant increase is found at 2 hours after birth compared to 15 minutes after birth only in the HFOV group (p=0.04). Afterwards AP50 remains stable like in the CV group. AP50 is statistically significant lower, reflecting more complement activation in the CV group than in the HFOV group at 6 and 8 hours after birth (p=0.04).

Total number of leukocytes (figure 1b) show a gradual increase during the period of ventilation. In the first 2 hours after birth total number of leukocytes increases in the HFOV group whereas it decreases in the CV group. However the differences are not statistically

significant.

PMNs (figure 1c) are low 15 minutes after birth in both groups (maternal sheep values of PMNs are 3500/ μ l) and increase gradually. A significant increase is already found at 2 hours after birth compared to 15 minutes after birth in both groups (CV group $p=0.03$, HFOV group $p=0.04$) and remains significantly higher during the entire ventilation period compared to 15 minutes after birth. Between the groups there are no statistically significant differences.

β -glucuronidase (figure 1d) is high 15 minutes after birth in both groups (maternal sheep values of β -glucuronidase are 0.05 OD/PMN) and decreases gradually. A significant decrease is found at 2 hours after birth in both groups (CV group $p=0.04$, HFOV group $p=0.05$) and remains significantly lower during the entire ventilation period compared to 15 minutes after birth. Between the groups there are no statistically significant differences.

Platelet function analysis (figure 1e) shows normal values during the first 4 hours after birth in both groups. In the CV group after 4 hours large differences are found in individual lambs. The values in the HFOV group remain stable. Platelet function value is significantly higher in the CV group than in the HFOV group at 8 hours after birth ($p=0.01$), reflecting better platelet function in the HFOV group than in the CV group.

The aPTT (figure 1f) in plasma is normal 15 minutes after birth and increases significantly in the first 2 hours after birth in both groups (CV group $p=0.04$, HFOV group $p=0.01$). In the next 6 hours aPTT decreases in both groups to values in the normal range, but in the HFOV group aPTT remains statistically significant higher during the entire ventilation period compared to 15 minutes after birth. Between the groups there are no statistically significant differences.

Thrombin time (data not shown) in plasma remains in the normal range (mean values 11.2-15.3 sec) during the entire ventilatory period in both groups. However within the HFOV group thrombin time increases significantly in the first 2 hours after birth ($p=0.008$) compared to 15 minutes after birth. Between the groups there are no statistically significant differences.

Thrombin inhibition (data not shown) in plasma is lower (mean values 1.33-1.40 IU/ml in both groups) than maternal values (1.45-1.5 IU/ml) 15 minutes after birth in both groups but remains in the initial range. Between the groups there are no statistically significant differences.

Figure 1.

Concentrations of studied parameters in plasma of the CV (closed circles) and HFOV (open squares) ventilated preterm lambs at 15 minutes, 2, 4, 6 and 8 hours after birth.

Data are presented as mean with SEM. # $p < 0.05$ compared to 15 minutes after birth within the CV group (Wilcoxon signed-rank test). ¶ $p < 0.05$ compared to 15 minutes after birth within the HFOV group (Wilcoxon signed-rank test).

* $p < 0.05$ for CV group vs. HFOV group (student's t -test).

a: AP50 (IU/ml), **b:** Number of leukocytes (number/ μ l), **c:** Number of PMNs (number/ μ l), **d:** β -glucuronidase (corrected for number of PMNs, OD/PMN), **e:** platelet function analysis (seconds), **f:** aPTT (seconds, in the HFOV group 2 lambs are excluded because of immeasurable aPTT due to sampling error)

The figures 2a-2c show measurements in BALF of elastase, thrombin and protein

corrected for urea in BALF.

Elastase (figure 2a) in BALF is low just before birth ($t=0$) in both groups and increases significantly in the first 2 hours after birth in both groups (CV group $p=0.01$, HFOV group $p=0.006$). Between the groups there are no statistically significant differences.

Thrombin activity (figure 2b) in BALF can be detected in both groups. In the HFOV group the value of thrombin is statistically significant higher at 4 hours after birth compared to $t=0$ ($p=0.004$). Afterwards it decreases to the same value as $t=0$. Between the groups there are no statistically significant differences.

Protein (figure 2c) in BALF shows the same tendency as elastase in BALF. Protein increases significantly in the first 2 hours after birth in both groups (CV group $p=0.04$, HFOV group $p=0.003$) and remains stable afterwards. Between the groups there are no statistically significant differences.

Figure 2.

Concentrations of study parameters in BALF of the CV (closed circles) and HFOV (open squares) ventilated preterm lambs at $t=0$ (just before delivery and after intubation), 2, 4, 6 and 8 hours after birth, corrected for urea concentrations in BALF.

Data are presented as mean with SEM. # $p < 0.05$ compared to $t=0$ within the CV group (Wilcoxon signed-rank test). ¶ $p < 0.05$ compared to $t=0$ within the HFOV group (Wilcoxon signed-rank test).

a: elastase (IU/mg), **b:** thrombin (IU/mg), **c:** protein (IU/mg).

After analysis only AaDO₂ at t = 8 hours was considered suitable for multiple regression analysis, because the other clinical values were not linearly and independently related to one of the laboratory values. Multiple regression analysis was performed for AaDO₂ at t = 8 hours against all laboratory values and no significant correlations were found.

Discussion.

To investigate the sequence of events in local as well as systemic activation of inflammation and clotting in RDS, and to compare the impact of different ventilatory modes (CV vs. HFOV) on the activation of inflammation and clotting, we studied preterm lambs of 132 days. We found activation of inflammation and clotting within 15 minutes after birth as well in the CV as in the HFOV group. HFOV possibly attenuates activation, but the results are not conclusive.

In a pilot study (n=3) we ventilated the first born lamb with CV and used the second born lamb as a control. We found that these lambs suffered from such a severe RDS that without ventilation severe respiratory acidosis (mean pH: 6.76, mean PaCO₂: 16.8 kPa) developed and the lambs died within about 1 hour. Therefore the study protocol was re-designed to compare CV with HFOV. Adequate ventilation was possible without surfactant administration. Laboratory investigations concerning inflammation and clotting systemically (in plasma) as well as locally (in BALF) yielded adequate reproducible results. So we concluded that the preterm lamb of 132 days gestational age provide a good animal model to perform these studies.

We found complement activation, low number of PMNs and a high level of β -glucuronidase in plasma already 15 minutes after birth in both groups. These findings suggest that systemic inflammation has possibly occurred before or very soon after birth with subsequent pooling of PMNs amongst others in the lungs. Local activation of inflammation appears likely from the observation of increasing levels of elastase in BALF in the first 2 hours after birth, remaining stable afterwards. In this experiment we did not determine inflammatory cells in BALF, this is planned in the next experiment. However, elastase in BALF probably better reflects inflammatory reaction in alveoli than the number of inflammatory cells itself.^[26] The finding of activated inflammation is in accordance with previous studies, which showed systemic activation of inflammation in RDS.^[1,5] A correlation between the severity of the inflammatory reaction (reflected by complement activation, decrease of PMN in the blood and release of β -glucuronidase) and the severity of protein leakage in the lungs was demonstrated in preterm rabbits.^[16] The same authors found a correlation between the severity of the inflammatory reaction and the clinical severity of RDS in preterm infants.^[27] Wagner et al. found complement activation in RDS patients, that was more pronounced in patients with a poor response to surfactant administration.^[28] In addition, Carlton et al. described systemic effects of RDS: they found that PMNs disappeared from blood due to sequestration in the lungs of preterm lambs with RDS, causing increased vascular permeability.^[29] This effect could be attenuated in neutropenic

lambs. Based on these studies, it was concluded that inflammation plays an important role in the pathogenesis of RDS as well as in the pathogenesis of the complications of RDS or bronchopulmonary dysplasia.

Clotting also plays an important role in the pathogenesis of RDS. We know fibrin deposits are found within the lung's intravascular, interstitial and intra-alveolar spaces in RDS.^[12] It is also known that platelets play an important role in inflammation. After platelet activation, platelet derived growth factor is released, P-selectin (necessary for PMN rolling on endothelial wall) is expressed and histamine is released, causing vasodilatation and increased vascular permeability.^[30] Therefore we studied activation of clotting in this study. Within 2 to 4 hours after birth activation of clotting was found in both groups, reflected by deteriorating platelet function 4 hours after birth, prolonging of aPTT within 2 hours, and lower thrombin inhibition in blood. The prolonged aPTT reflects factor VIII consumption due to clotting activation in plasma. Local activation of clotting appeared likely from the demonstration of thrombin activity in BALF, since thrombin is not found in normal lungs. However, because protein in BALF also increases in the first hours after birth, we can not exclude the possibility that the thrombin activity in BALF is the result of capillary leakage. These findings suggest that systemic as well as local activation of clotting take place after birth. Because activation of clotting and platelet activation are found later than activation of inflammation, we consider activation of clotting is not causative in RDS, but is activated secondary to activation of inflammation.

HFOV is a mode of ventilation considered less damaging to human preterm lung than CV.^[18,19] Therefore, we compared CV to HFOV. There seemed to be less complement activation in the HFOV group, reflected by significantly less decreased AP50 levels than in the CV group after 6 hours of ventilation. Both CV and HFOV ventilated animals showed a significant increase of elastase in BALF after 2 hour of ventilation compared to just before birth. Although elastase levels in BALF from lambs ventilated with CV were not statistically significant higher than elastase levels in BALF from lambs ventilated with HFOV the tendency towards higher elastase levels in HFOV ventilated lambs is in accordance with the results in blood, suggesting a protective effect of HFOV. Other investigators described similar results. Imai et al. found in lung-lavaged rabbits less release of chemical inflammatory mediators in BALF and less lung injury in the rabbits ventilated with HFOV than ventilated with CV.^[31] In contrast, Thome et al. found no beneficial effect in patients that were treated with HFOV compared to those treated with conventional ventilation with respect to concentrations of albumin and pro-inflammatory cytokines in BALF.^[32] However it could be argued that in that study, the BALF collected during the first day was pooled and maybe differences between the two groups have disappeared already after 12 or more hours after birth.

In this study, investigated parameters concerning clotting showed only for platelet function a statistically significant difference between HFOV and CV, suggesting a protective effect of HFOV on the activation of clotting. Although no significant differences were found, the trend of aPTT and thrombin inhibition, as well as thrombin in BALF, further support this

hypothesis.

We conclude that the signs of systemic activation of inflammation, especially activation of PMNs and release of β -glucuronidase can be found in the ventilated preterm lamb with RDS within 15 minutes after birth. Pooling of PMNs in the lungs leads to subsequent release of elastase. Afterwards, or due to activation of inflammation, clotting is activated systemically as well as locally. The activation takes place in the lambs ventilated with CV as well as in the lambs ventilated with HFOV, but HFOV possibly attenuates activation. Further research directs to the very early, i.e. in the first 15 minutes after birth, activation of inflammation. Once we have more insight in the initial steps in the cascade, therapeutic interventions, for example surfactant instillation, can be evaluated.

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Activation of the inflammatory reaction within minutes after birth in ventilated preterm lambs with neonatal RDS.

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Abstract.

To study activation of the inflammatory reaction within minutes after birth, we measured parameters of inflammation before and immediately after birth.

To assess whether RDS or birth itself initiates activation, we compared preterm ventilated lambs with term non-ventilated lambs.

Preterm lambs were delivered by cesarean section at 132 days gestational age (term 145 days) and were ventilated by conventional ventilation (n=9). Before clamping the cord, 5, 10 and 15 minutes after birth, blood was sampled from umbilical catheters. Term lambs (n=9) were born spontaneously after 140-145 days gestational age. Immediately after birth a venous umbilical catheter was inserted. Blood was sampled before the first breath and 5, 10, 15 and 20 minutes after birth while the lamb was breathing spontaneously. Blood was analyzed for AP50 (complement activation), number of polymorphonuclear leukocytes (PMNs) and β -glucuronidase (released from activated PMNs).

In preterm lambs we found decreased number of polymorphonuclear leukocytes and increased levels of β -glucuronidase already at 5 minutes after birth. In the term lambs we found only short-term mild decrease of PMNs and short-term increase of β -glucuronidase.

We conclude that systemic activation of the inflammatory reaction can be found in ventilated preterm lambs with RDS within 5 minutes after birth. This very early activation is mild, transient and less pronounced in term born spontaneously breathing lambs compared to preterm, ventilated lambs with RDS.

Introduction.

Neonatal Respiratory Distress Syndrome (RDS) is a disease of preterm infants, due to structural immaturity of the lungs and immaturity of the pulmonary surfactant metabolism, resulting in surfactant deficiency and surfactant dysfunction. Apart from these factors there is increasing evidence that inflammation contributes to the progression of this disease. An enhanced inflammatory response in bronchoalveolar lavage fluid as well as in plasma in infants with RDS developing bronchopulmonary dysplasia was described.^[1,2] However most authors describe abnormalities in bronchoalveolar lavage fluid and plasma that can be found on various days after birth, probably more reflecting the role of the inflammatory reaction in the progression from RDS to bronchopulmonary dysplasia than the role of inflammation in RDS itself.^[3,4]

In a previous study we found systemic activation of the inflammatory reaction at 15 minutes after birth in ventilated preterm lambs with RDS.^[5] However, the first blood sample in that study was taken 15 minutes after delivery; therefore it was unknown whether the inflammatory reaction was activated due to ventilation or due to delivery or already existed before delivery. To study the exact moment of activation of the inflammatory reaction in lambs with RDS, we now studied preterm lambs of 132 days gestational age with RDS, before and immediately after delivery. To assess the contribution of birth itself as a physiologic activator of the inflammatory reaction, we included term born non-ventilated lambs as controls.

Methods and materials.

The experiments on preterm lambs were performed in the central animal laboratory of the University of Groningen, under approved animal care protocols of the University of Groningen with concern for animal welfare. The experiments on term lambs were performed in the Dutch center for experimental cattle breeding (ID-Lelystad). We studied 9 preterm lambs of 132 days gestational age and 9 term lambs (140-145 days gestational age).

Treatment of the animals.

Preterm lambs were delivered by cesarean section at 132 days gestational age. Before delivery an umbilical arterial catheter was inserted, blood was sampled and the lambs were intubated orotracheally with a cuffed 5.5-mm ID tube and were suctioned endotracheally once to remove excess lung water or secretions. The next step was to clamp and cut the umbilical cord and after drying and weighing, the lambs were mechanically ventilated with a time-cycled pressure-limited infant ventilator (Babylog 8000, Dräger, Lübeck, Germany).

Ventilator settings were immediately adjusted to achieve a tidal volume of 5-8 ml/kg and an expiratory minute volume of about 300 ml/kg/min. Inspiratory oxygen concentration was adjusted to maintain transcutaneous O₂ saturation between 92 and 95% (measured with Pulseoximeter Nellcor, Hayward, California, USA).

In preterm lambs, the arterial umbilical catheter was used for blood sampling (immediately after inserting (before delivery (t=0)) and 5, 10 and 15 minutes after initiating ventilation) and measurement of blood pressure. Patency was maintained by continuous infusion of saline solution 0.9%. An additional catheter was advanced in the vena cava inferior through the umbilical vein and was used for the administration of fluids (3 ml/kg/h), glucose (5 mg/kg/min) and sodium pentobarbital (Nembutal) for sedation. Body temperature was measured by a rectal thermometer and was kept at 38°C by using preheated mattress and blankets.

Term lambs were born spontaneously. After birth of the lambs' head, covering nose and mouth with a surgical glove prevented breathing. Immediately after birth of the lambs' body, a venous umbilical catheter was inserted in a sterile way and blood was sampled (zero-sample). This procedure took about 2 minutes and thereafter the lamb was stimulated to breathe spontaneously. Every 5 minutes blood was sampled for the first 20 minutes after birth (5 samples). Afterwards the catheter was removed and the umbilical cord was disinfected and ligated.

Assays.

In ventilated animals at t=15 minutes after birth, 0,4 ml heparinized blood was used for blood gas analysis to assess PaO₂, PaCO₂, and pH, using an ABL 330 blood gas analyzer (Radiometer Co, Copenhagen, Denmark) and to assess SaO₂ using an OSM-3 analyzer (Radiometer Co, Copenhagen, Denmark).

From all samples, 0.25 ml blood was anticoagulated with EDTA 0.01 M and was used to prepare Giemsa stained blood smears to determine by light microscopy total leukocyte count and differential cell counts to determine the number of polymorphonuclear leukocytes (PMNs). The remaining blood was anticoagulated immediately with citrate 0.31% and placed on ice. It was used to determine AP50 and β -glucuronidase.

AP50 titer was used as a measurement of activation of the alternative route of the complement system. A reduction of AP50 indicates in vivo complement activation.

β -glucuronidase activity was used as a marker of the inflammatory reaction. β -glucuronidase is released from azurophilic granules from PMNs after activation.^[6] β -glucuronidase amount was measured according to Baehner and was expressed as amount (in optical density [OD]) corrected for the number of PMNs.^[7]

Data and statistical analysis.

Clinical data of preterm lambs (ventilatory settings, blood gas values, oxygenation index and alveolar-arterial oxygen difference) are presented as mean and standard deviation (SD). Laboratory data are presented as mean and standard error of the mean (SEM) in the table and the figures. For statistical analysis Wilcoxon signed-rank test was used to determine changes between the first sampling point and successive points.

Student's t-test was used to determine differences between groups. Tests were considered significant if $p < 0.05$.

Results.

Ventilatory data for the preterm lambs are given in table 1.

t = 15 min	
FiO ₂	0.76 ± 0.15
Mean airway pressure	12.8 ± 1.7 cm H ₂ O
PaO ₂	30.7 ± 16.8 kPa
PaCO ₂	8.2 ± 1.1 kPa
Oxygenation index (OI)*	6.8 ± 6.7
Alveolar-arterial O ₂ difference **	236 ± 173

Table 1.

Ventilatory characteristics and blood gas analysis values for the preterm ventilated lambs. Data are presented as mean ± SD.

*OI = $P_{aw} \times FiO_2 / PaO_2$

** alveolar-arterial O₂ difference = $FiO_2 (P_{atm} - P_{H_2O}) - PaO_2 - PaCO_2$

Both AP50 (figure 1a) before birth in preterm lambs and AP50 in term lambs immediately after birth and before the first breath (t=0) were significantly lower than maternal values. AP50 in term lambs was significantly higher than in ventilated preterm lambs with RDS ($p=0.029$) at t=0. AP50 in as well preterm as term lambs showed no significant change during the first 20 minutes after birth.

PMNs (figure 1b) in preterm lambs before birth were significantly lower than maternal values. PMN count in term lambs at t=0 was also significantly lower than maternal values but was significantly higher than in ventilated preterm lambs with RDS and remained higher in the first 15 minutes after birth ($p < 0.01$). PMNs in preterm lambs decreased, and at 5, 10 and 15 minutes after birth PMNs were significantly lower than at t=0 ($p=0.008$). To make this decrease more clear, the exact numbers of PMNs are also given in table 2. In contrast to ventilated preterm lambs with RDS, PMNs in term lambs showed only a slight, not statistically significant decrease after birth.

	Preterm lambs	Term lambs		p
t=0	277 (46)	1997 (396)	Number/ μ l	< 0.01
t=5	152 (42)	1692 (381)	Number/ μ l	< 0.01
t=10	123 (39)	1453 (389)	Number/ μ l	< 0.01
t=15	120 (48)	1532 (374)	Number/ μ l	< 0.01
t=20	Not determined	1547 (367)	Number/ μ l	

Table 2.

Number of PMNs (mean and SEM).

* P-value reflects statistical significance for difference between term and preterm lambs.

β -glucuronidase (figure 1c) before birth in preterm lambs and in term lambs at t=0 was not significantly higher than maternal values. In preterm lambs at t=0, however, it was significantly higher than in term lambs. β -glucuronidase in preterm lambs increased and was significantly higher at 5, 10 and 15 minutes after birth compared to t=0 (p=0.03 at 5 minutes and p=0.008 at 10 and 15 minutes compared to t=0). β -glucuronidase in term lambs showed a statistically significant increase 10 minutes after birth compared to t=0 (p=0.01) and decreased again in the next 10 minutes.

Figure 1a. **Figure 1b.** **Figure 1c.** **Figure 1.**

Measurements in plasma of the CV (closed circles) preterm lambs before clamping the cord and at 5, 10, 15 minutes after birth and of the term lambs (open triangles) immediately after birth and before the first breath and at 5, 10, 15 and 20 minutes after birth and of the mothers (open diamond). Data are presented as mean with SEM. * $p < 0.05$ and ** $p < 0.01$ for term group vs. preterm group (student's t -test).

$p < 0.05$ and ## $p < 0.01$ compared to t=0 (before birth) within the preterm group, † $p < 0.05$ compared to t=0 within term group (Wilcoxon signed-rank test).

a: AP50 (IU/ml), **b:** Number of PMNs (number/ μ l), **c:** β -glucuronidase (corrected for number of PMNs, OD/PMN).

Discussion.

To study the early process of the inflammatory reaction in preterm lambs with RDS, we focused on the first 15 minutes after birth of 9 preterm lambs of 132 days gestational age. In a previous study we found systemic activation of the inflammatory reaction at 15 minutes after birth in ventilated preterm lambs with RDS.^[5] In this study we demonstrated that systemic activation of the inflammatory reaction takes place within 5 minutes after preterm birth, since the number of PMNs decreased and β -glucuronidase levels increased significantly within 5 to 10 minutes after birth in ventilated preterm lambs with RDS.

We used the drop in PMNs as a marker for activation of the inflammatory reaction. In normal subjects no or only a few PMNs can be found in the alveoli. In lungs of patients with respiratory problems, numerous PMNs can be found in alveolar spaces and PMNs are considered to play an important role in the defense against microorganisms or other lung damage.^[8,9] The lung vasculature contains a large reservoir of PMNs (the margined pool). In response to complement factors or endotoxins or interleukins, secretory products of phagocytes and platelet-derived products, PMNs roll over and adhere to the endothelium. This is the first step towards migration of PMNs through the endothelium in the alveolar lumen.^[8] It would be possible that the drop in PMNs we found in the preterm lambs was a normal transition phenomenon, reflecting the forming of a margined pool in the lung vasculature after delivery due to increase in pulmonary blood flow. However one would expect the same (or an even larger) drop in number of PMNs in the term lambs, which was not found. The drop in PMNs therefore is considered as an acute result of systemic activation with aggregation of PMNs and extravasation on the place of damage, in this case the lungs. We measured so fast after the after starting ventilation and after the beginning of RDS that compensatory release of PMNs from the bone marrow was actually impossible.

To study birth as possible etiological factor for the activation of the inflammatory reaction, we included full term spontaneously born lambs. The number of PMNs is significantly higher in term lambs than in preterm lambs and the term lambs did not show a significant decrease in number of PMNs. The percentage decrease in preterm lambs was 45%, 55% and 60% at 5,10,15 minutes compared to $t=0$ where a percentage decrease in term lambs of 19%, 29% and 15% at 5,10, 5 minutes compared to $t=0$ was found. Therefore we conclude that, although there is a decrease in PMNs, the decrease is significantly less striking than in preterm lambs. This reflects a moderate and transient activation of the inflammatory reaction in term lambs, which is not persistent as it is in preterm lambs. We observed also a significant increase in the β -glucuronidase level in term lambs that disappeared after 10 minutes in contrast to the persistent increase in β -glucuronidase level that was demonstrated in preterm ventilated lambs. The percentage increase in preterm lambs was 174% compared to 72% in term lambs. These findings suggest that birth itself is not responsible for the persistent activation of the inflammatory reaction that we observed in preterm ventilated lambs with RDS.

Early activation of inflammation in RDS has been described before, although not as early as in this study. Previous studies have shown a positive correlation between severity of neutropenia in the first hours after birth and severity of RDS in the forthcoming hours and days as well in lambs as in preterm infants.^[10-12] The question arises if it is mechanical ventilation or RDS or a combination that induces activation of inflammation. To investigate whether ventilation contributes to activation of the inflammatory reaction it would be very interesting to study term lambs with healthy lungs, born by cesarean section, ventilated from birth. Positive pressure ventilation itself can induce lung injury and alveolar inflammation. Possible causative mechanisms are repetitive opening and closing of atelectatic lung units, shear forces and cyclic stretch.^[13,14] However, most of these studies have been performed in models of acute lung injury or in epithelial cell linings and not in ventilated animals or humans with healthy lungs. Models of acute lung injury always induce activation of the inflammatory reaction just by the procedure causing lung injury. One study compared the combined effects of hyperoxia and mechanical ventilation in term healthy newborn pigs to unventilated controls. Clinical signs of lung injury were seen and marked inflammation was found after pathological analysis of lung sections after ventilation healthy lungs with 100% oxygen.^[15]

Carlton et al. have hypothesized that the pattern of lung expansion immediately after birth and/or oxygen supplementation after preterm birth influences PMN sequestration, suggesting that a combination of RDS and ventilation induces activation of inflammation.^[11] Epithelial damage and increased permeability of the alveolar capillary membrane due to these mechanisms lead to IL-8 gene expression and IL-8 production, which is a potent chemotactic cytokine.^[9] The recruited intravascular PMNs aggregate and adhere to the vascular endothelium and migrate to the alveolar space where they become further activated. All these steps are also influenced by cytokines such as IL-8. This subsequently leads to augmented vascular permeability, enhanced PMN adhesion and activation contributing further to acute lung injury. Lung protective strategies with the use of low tidal volumes have been proven effective in attenuating the inflammatory reaction.^[16] Especially the use of high volumes is described as a cause of lung injury and a cause of release of inflammatory mediators in the lung and the systemic circulation.^[17,18]

Oxygen supplementation is another mechanism that has been suggested to activate inflammation.^[8,11,19,20] Ventilation of the preterm lung with deficient antioxidant enzymes, especially ventilation with oxygen, will induce the formation of oxygen radicals which play an important role in host defense, but which on the other hand can serve as primary mediators of inflammation and can induce lung injury. We ventilated the lambs with relatively high concentrations of inspiratory oxygen to obtain oxygen saturation between 92 and 95%. This resulted however in high pO₂ levels at 15 minutes after delivery. However the activation of the inflammatory reaction we found in preterm ventilated lambs is not likely due to oxygen, considering the very short time span of oxygen exposure. Pitkanen described that oxidant stress and its effects increase after birth and reach a maximum by 2 to 6 days.^[19]

Although AP50 and PMNs in term lambs were lower than maternal values, they were higher than in preterm ventilated lambs with RDS, so we consider this not as a sign of activation

of the inflammatory reaction in term lambs. This finding is in concordance with studies in humans that showed that the complement levels as well as total number of leukocytes (and especially PMNs) are significantly reduced in preterm and full-term neonates when compared with adult levels and that they correlate significantly with gestational age.^[21,22] In comparison to the preterm lambs, term lambs showed only a slight and temporary decrease in number of PMNs, which indicates there is less (persistent) activation of the inflammatory reaction than in the preterm lambs. Term lambs also showed an increase in β -glucuronidase 10 minutes after birth, which recovered again within another 5 minutes. This was in contrast with the increase in β -glucuronidase in ventilated preterm lambs already within 5 minutes after birth, which remained high. This temporary increase of β -glucuronidase possibly reflects an initiation of activation of the inflammatory reaction, which is discontinued spontaneously in term lambs in contrast to preterm lambs with RDS and ventilation. We hypothesize that in term lambs the ongoing activating mechanism is lacking or there is effective inhibition of activation of the inflammatory reaction, whereas in preterm lambs the activation of the inflammatory reaction continues or the inhibiting mechanism has not been developed adequately yet.

We conclude that systemic activation of the inflammatory reaction takes place within 5 minutes after birth. After term birth, the inflammatory reaction is less pronounced and less persistent activated than after preterm birth, followed by ventilation for RDS. We hypothesize that artificial ventilation of preterm lungs with RDS might be responsible for the persistent activation of the inflammatory reaction. Because of the immediate activation of the inflammatory reaction after birth and the relationship with RDS and chronic lung disease, therapeutic interventions for prevention of secondary injury after preterm birth have to be directed at the period immediately after birth (within the first few minutes) or preferably before birth.

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Antenatal glucocorticoids attenuate activation of the inflammatory reaction and clotting in preterm lambs.

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Abstract.

Recently we have shown that activation of inflammatory reaction and clotting can be found immediately after delivery in preterm lambs ventilated for respiratory distress syndrome (RDS).

To investigate whether antenatal glucocorticoids would attenuate postnatal activation of the inflammatory reaction and clotting, we studied ventilated preterm lambs, delivered by cesarean section, 24 hours after antenatal betamethasone or placebo.

Blood was sampled before clamping the cord, 5,10,15 minutes after delivery and 2-hourly afterwards. Blood was used to determine oxygenation index, AaDO₂, AP50, polymorphonuclear leukocytes (PMNs), β -glucuronidase, thrombin inhibition, aPTT and clot lysis time. Broncho-alveolar lavage fluid was sampled before clamping the cord, 30 minutes and 1,2,4,6,8 hours after delivery, and was analyzed for elastase, thrombin and protein. After removal of the lungs, static compliance and water content of the lungs were determined.

We found that betamethasone lambs had lower oxygenation index and AaDO₂ than controls. At birth, PMNs were higher and β -glucuronidase was lower after betamethasone treatment. PMNs and β -glucuronidase did not change in betamethasone lambs, in contrast to controls. Thrombin inhibition, aPTT and clot lysis time did not change in betamethasone lambs, in contrast to controls. In both groups, elastase and protein in broncho-alveolar lavage fluid increased, thrombin increased in controls. Static compliance was better and water content of the lung was lower in betamethasone lambs.

We conclude that early systemic activation of the inflammatory reaction and clotting in preterm lambs with RDS is attenuated by antenatal betamethasone administration. Whether this is a direct effect of betamethasone on the inflammatory reaction or a result of reduced ventilatory support because of less severe RDS after antenatal betamethasone remains to be elucidated.

Introduction.

Neonatal respiratory distress syndrome (RDS) is a disease of preterm infants characterized by surfactant deficiency and structural immaturity of the lungs. We recently demonstrated that the number of polymorphonuclear leukocytes (PMNs) decreased and the amount of β -glucuronidase increased in preterm lambs with RDS within minutes after birth, which we interpreted as systemic activation of the inflammatory reaction.^[1] We also demonstrated activation of clotting.^[1] We assumed that activation of the inflammatory reaction and clotting immediately after birth play an additional role in the development of RDS.

Maternal administration of glucocorticoids has been shown to decrease the incidence of RDS in preterm infants.^[2,3] Several mechanisms have been proposed to explain the beneficial effects of antenatal glucocorticoids on the development of RDS. Glucocorticoids are assumed to induce pulmonary maturation by up-regulating genes that are involved in the synthesis of surfactant proteins and phosphatidylcholines.^[3,4] Glucocorticoids also have an effect on lung cell differentiation and on remodelling of the alveolar architecture including thinning of the interstitial components of the lung.^[4,5] Glucocorticoids mediate water reabsorption in the perinatal lung by influencing the expression of aquaporin-1 channel proteins in the lung.^[6]

Early postnatal steroids are known to minimize lung inflammation as has been demonstrated by Wang et al.^[7] However, the anti-inflammatory effects of antenatal glucocorticoids and the consequence of attenuation of early inflammation on the development of RDS are unknown.^[5] Possibly the anti-inflammatory effect of glucocorticoids is another reason for the beneficial effect of antenatally administered glucocorticoids on postnatal lung function in preterm infants. In this study we investigated whether antenatal glucocorticoids could attenuate the change in PMNs, β -glucuronidase and the activation of clotting immediately after birth and whether this would lead to improved lung function and decreased need for ventilatory support.

Methods and materials.

The experiments were performed in the central animal laboratory of the University of Groningen, under approved animal care protocols of the University of Groningen with concern for animal welfare.

Treatment of the animals.

We planned a cesarean section at 132 days gestational age (term is 145 days). Twenty-four hours before the planned cesarean section, ewes who were randomized for betamethasone treatment (by coin method) were injected with 0.5 mg/kg betamethasone. In all ewes a cesarean section through a lateral incision was performed under sedation with Sevoflurane inhalation and with the use of subcutaneous prilocaine for local anesthesia. Before clamping the umbilical cord, lambs were intubated orotracheally with a cuffed 5.5-mm ID tube and the airways were suctioned once and an umbilical arterial catheter was inserted for blood sampling. After delivery the lambs were dried and weighed and were mechanically ventilated with a time-cycled pressure-limited infant ventilator (Babylog 8000, Dräger, Lübeck, Germany) during 8 hours, while blood samples were taken. Initial ventilator settings were: frequency 60/min, inspiratory time and expiratory time 0.5 sec., gas flow 8 l/min. Peak inspiratory pressure was initially set at 20 cm H₂O and adjusted instantly to achieve a tidal volume of 5-8 ml/kg and an expiratory minute volume of about 300-350 ml/kg/min. Positive end-expiratory pressure was set at 4 cm H₂O. Fractional concentration of inspired oxygen (FiO₂) was set at 0.40 and adjusted to maintain transcutaneous O₂ saturation between 92 and 95% (measured with Pulseoximeter Nellcor, Hayward, California, USA) and partial pressure of arterial O₂ (PaO₂) between 6.0 and 8.0 kPa. During the study peak inspiratory pressure was adjusted to achieve partial pressure of arterial CO₂ (PaCO₂) values of 5.5 to 7.0 kPa. The lambs were ventilated in lateral lying position, which was changed to the opposite side every 2 hours.

In all lambs the umbilical arterial catheter was used for blood sampling and measurement of blood pressure. Patency was maintained by continuous infusion of saline solution 0.9% at a rate of 2.0 ml/h. During the study, blood pressure was maintained in the normal range (mean 40 mmHg). A second catheter was advanced in the vena cava inferior through the umbilical vein and was used for the administration of fluids (3 ml/kg/h), glucose (5 mg/kg/min) and sodium pentobarbital. Serum glucose levels were measured and were kept in the normal range (4-9 mmol/l) by adjusting the amount of glucose that was infused. Body temperature was measured continuously by a rectal thermometer. Body temperature was kept at 38°C by using preheated mattress and blankets. Sodium pentobarbital (Nembutal®) was given for sedation, initially 15 mg/kg/h; dosage was adjusted according to activity, blood pressure and heart rate of the lamb.

After 8 hours the study was ended, the lamb was killed with an overdose sodium pentobarbital, and a thoracotomy was performed to remove the lungs.

Blood sampling.

Heparinized blood samples were obtained hourly for blood gas analysis to assess PaO₂, PaCO₂, and pH, using an ABL 330 bloodgas analyzer (Radiometer Co, Copenhagen, Denmark). Immediately after inserting the umbilical arterial catheter (t=0), at 5, 10, 15 minutes after birth and at 2 hour intervals afterwards, a blood sample was obtained. The blood was anticoagulated immediately with citrate 0.31% and placed on ice. After centrifuging at 1100 g for 10 minutes, the plasma was stored at -80°C until AP50, β -glucuronidase, activated partial thromboplastin time (aPTT), thrombin inhibition and clot lysis time were determined.

0.25 ml blood was anticoagulated with EDTA 0.01 M and was used to determine leukocyte count, and to make blood smears for differential cell counts, especially to determine the number of PMNs.

Bronchoalveolar lavage.

Immediately after intubation (t=0) and 30 minutes, 1,2,4,6 and 8 hours after delivery bronchoalveolar lavage was performed by injecting 1 ml/kg saline solution 0.9% intratracheally with immediate aspiration, this was repeated three times. The bronchoalveolar lavage fluid (BALF) was centrifuged at 1100 g and the supernatant was stored at -80°C until further processing took place. In BALF elastase, thrombin and protein concentrations, corrected for urea, were determined.

Measurement of clinical condition.

Severity of RDS was expressed as oxygenation index (Mean airway pressure x FiO₂/ PaO₂), alveolar-arterial partial O₂ tension difference ($AaDO_2 = FiO_2 (P_{atm} - P_{H_2O}) - PaCO_2 - PaO_2$) and dynamic compliance (dV/dP/bodyweight).

To express the ventilatory support, we used the ventilator efficiency index ($3800/[\Delta P \times \text{frequency} \times PaCO_2]$).^[8]

Blood assays.

AP50 titer (the alternative pathway hemolytic activity assay as a measure of activation of alternative route of complement system) is the plasma dilution that causes 50% lysis of non-sensitized rabbit erythrocytes in a standard suspension after prevention of activation of the

classical route with EGTA and Mg^{2+} .^[9] A reduced value indicates more complement consumption due to activation.

Determination of total leukocyte count from EDTA blood was performed microscopically using a chamber after a gentian violet staining. Giemsa stained smears were used to determine differential cell counts. Total number of PMNs was calculated from these two measurements.

β -glucuronidase activity was used as a marker of the inflammatory reaction. β -glucuronidase is released from azurophilic granules from PMNs after activation.^[10] β -glucuronidase amount was measured according to Baehner and was expressed as amount (in optical density [OD]) corrected for the number of PMNs.^[11]

The activated partial thromboplastin time (aPTT, a screening test for abnormalities in the intrinsic clotting system, especially factors VIII, IX, XI, XII, X and II, and prekallikrein, high molecular weight kininogen and fibrinogen) was measured by adding 100 μ l aPTT reagent (Sigma Diagnostics, St. Louis, USA) and 100 μ l calciumchloride to lamb plasma and recording clotting time.

Thrombin inhibition (a method to determine the amount of antithrombin III in plasma) was measured according to the test kit from Kabi (Kabi Diagnostics Mölndal, Sweden), with modifications adapted to sheep plasma. A higher OD value at 405 nm using the microplate reader indicates a higher residue of thrombin, reflecting a lower antithrombin III concentration in the tested plasma.

Clot lysis time was determined by adding thrombin to the test plasma and measuring the time needed to resolve a clot that has been formed. A shorter clot lysis time reflects a more activated state of fibrinolysis.

BALF assays.

Elastase in BALF (released by activated PMNs in the lung) was measured by incubating BALF with elastase substrate (Omnilabo, Breda, The Netherlands) for three hours at 37°C.

Thrombin in BALF (a measurement of local activation of the clotting system in the lung) was measured after adding a thrombin specific substrate (Chromogenix S2238, Mölndal, Sweden). A higher OD value at 405 nm using the microplate reader indicates a higher level of thrombin.

Protein in BALF (a measurement of capillary leakage due to local injury in the lung) was measured according to Lowry et al.^[12] A higher OD value at 655 nm using the microplate reader indicates a higher protein level.

To correct for dilution, values of elastase, thrombin and protein were corrected for the measured urea in BALF. Urea was determined according to Marsh et al.^[13]

Static compliance.

After careful removal of the lungs, the lungs were controlled for air leakage. If this was not the case, static compliance was measured. The trachea was canulated and the lungs were connected to a closed system with pressure registration. After completing 3 complete inflations and deflations of the lungs with 60 ml of air, the lungs were inflated in steps of 5 cmH₂O pressure increments till a maximum pressure of 30 cmH₂O was reached and deflated stepwise afterwards. The corresponding volumes at each step were recorded. This procedure was repeated twice.

Lung water content.

Water content of the upper and lower lobes of the left lung was determined after determining static compliance. For determining lung water content, we sampled parts of the upper and lower lobe of the left lung of each lamb. After weighing, the samples were frozen at -80°C. After freeze-drying until a constant weight was reached, the samples were weighed again and water content was determined by subtracting the weights and relating them to the wet weight. Measurements were performed in duplicate.

Data and statistical analysis.

Continuous variables are presented as mean and standard error of the mean (SEM). Student's t-test was used to evaluate for statistical significant differences of measurements of clinical condition and measurements of activation of the inflammatory reaction and clotting between the two groups. Mann Whitney test was used to evaluate differences in lung function because data were not normally distributed and was used to evaluate differences in water content because of availability of small numbers of data. For statistical evaluation of changes between the first sampling moment and successive moments, Wilcoxon signed-rank test was used. Tests were considered significant if $p < 0.05$.

Results.

We studied 15 preterm lambs of 132 days gestational age without maternal betamethasone and 9 lambs of the same gestational age after maternal betamethasone administration. The

mean weight of the 2 groups was comparable (controls 3440 ± 160 grams, betamethasone group 3744 ± 276 grams).

Figures 1a-1d show severity of RDS and necessary ventilatory support. As is shown in the pictures, betamethasone treated lambs had less severe RDS than controls and betamethasone treated lambs needed less ventilatory support than controls.

Figure 1a.

Figure 1b.

Figure 1c.

Figure 1d.

Figure 1.

Measurements of severity of RDS and necessary ventilatory support of the controls (closed circles) and preterm lambs after antenatal betamethasone (open triangles) from 15 minutes to 8 hours after birth. Data are presented as mean with SEM.

* $p < 0.05$ and ** $p < 0.01$ for controls vs. betamethasone group. (Student's *t*-test).

$p < 0.05$ and ## $p < 0.01$ compared to $t = 15$ minutes within the control group. + $p < 0.05$ compared to $t = 15$ minutes within the betamethasone group. (Wilcoxon signed-rank test).

a: Oxygenation index, **b:** AaDO₂, **c:** dynamic compliance (ml/cm H₂O/kg), **d:** Ventilator Efficiency Index.

The figures 2a-2f show laboratory data of blood analysis including AP50, number of PMNs, β -glucuronidase (corrected for number of PMNs), residual thrombin activity aPTT and clot lysis time in plasma.

AP50 (figure 2a) was already low before birth in both groups compared to maternal sheep values (maternal values: 219 [betamethasone] respectively 298 IU/ml [controls], n.s.). In both groups of lambs AP50 decreased, reflecting complement consumption.

Total number of PMNs (figure 2b) was markedly higher in the betamethasone group than in the controls. PMNs showed a slight temporary increase in the first 5 minutes after birth in the betamethasone treated lambs in contrast to the controls who had a statistically significant decrease in the first 15 minutes. Maternal values were the same for the 2 groups.

β -glucuronidase (figure 2c) was significantly lower in the betamethasone group than in the controls. In the betamethasone group, β -glucuronidase showed no change, in contrast to the controls where it increased. Maternal values were the same for the 2 groups.

Residual thrombin activity in plasma (figure 2d) was lower in the betamethasone group than in the controls. Thrombin activity showed no change in the betamethasone group, whereas in the controls it decreased significantly. We used the pattern and comparisons of thrombin inhibition merely as a method to compare the phenomenon of clotting in the groups than as an exact measurement. We considered the lower residual thrombin activity after betamethasone treatment as a reflection of increased availability of ATIII. We have no reasons to assume that the capacity of the liver to produce ATIII would be different between the groups. Values of ewes after betamethasone treatment were significantly lower than values of control ewes. Because we did serial measurements, a change in residual thrombin activity can be interpreted as a change in ATIII. After betamethasone treatment no change was detected, we considered this a reflection of no activation of clotting and anticoagulation.

The aPTT (figure 2e) in plasma was measured from 15 minutes after birth. APTT was longer after antenatal betamethasone. Maternal values were the same for the two groups.

Clot lysis time (figure 2f) was longer in the betamethasone group than in the controls. Values of ewes after betamethasone treatment were also significantly longer than values of control ewes. Clot lysis time remained unchanged in the betamethasone group, whereas in the controls clot lysis time increased significantly. This means that fibrinolysis was not activated/stimulated in the betamethasone group, in contrast to controls.

Figure 2. (see next page)

Concentrations of markers of inflammation and clotting in plasma of the controls (closed circles) and preterm lambs after antenatal betamethasone (open triangles) before clamping the cord ($t=0$), 5,10,15 minutes and 2,4,6,8 hours after birth. Data are presented as mean with SEM.

* $p<0.05$ and ** $p<0.01$ for controls vs. betamethasone group. (Student's t -test).

$p<0.05$ and ## $p<0.01$ compared to $t=0$ within the control group. + $p<0.05$ and ++ $p<0.01$ compared to $t=0$ within the betamethasone group. (Wilcoxon signed-rank test).

a: AP50 (IU/ml), **b:** Total number of PMNs (number/ μ l), **c:** β -glucuronidase (corrected for number of PMNs, OD/PMN), **d:** Plasma thrombin inhibition, determined by measuring residual thrombin activity (IU/ml), **e:** aPTT (seconds), **f:** clot lysis time (hours).

Figure 2a.

Figure 2b.

Figure 2c.

Figure 2d.

Figure 2e.

Figure 2f.

The figures 3a-3c show laboratory data of BALF analysis including elastase, thrombin activity and protein.

Elastase (figure 3a) in BALF was low before birth ($t=0$), as was expected. In both groups elastase increased significantly, but in the betamethasone group the increase was less than in the controls.

Thrombin activity (figure 3b) in BALF was lower in the betamethasone group than in the controls. In the betamethasone group thrombin in BALF remained stable whereas in controls thrombin increased significantly. The lack of statistically significant difference between the two groups is due to the large variance within the control group.

Protein (figure 3c) in BALF showed the same pattern as elastase in the BALF. Static compliance was statistically significant better after betamethasone treatment throughout the complete inflation and deflation curve.

Mean water content as fraction of wet weight of lungs post mortem of the left upper lobe was 0.83 (SEM 0.01) in controls and 0.78 (SEM 0.02) in the betamethasone group (n.s.). Mean water content as fraction of wet weight of lungs post mortem the left lower lobe was significantly lower in the betamethasone treated group than in the controls (0.85 (SEM 0.01) in controls and 0.76 (SEM 0.03) in the betamethasone group, $p=0.006$).

Figure 3.

Concentrations of elastin, thrombin and protein in BALF corrected for urea of the controls (closed circles) and preterm lambs after antenatal betamethasone (open triangles) before clamping the cord ($t=0$), and 1,2,4,6,8 hours after birth. Data are presented as mean with SEM.

* $p<0.05$ for controls vs. betamethasone group. (Student's t -test).

$p<0.05$ and ## $p<0.01$ compared to $t=0$ within the control group. + $p<0.05$ and ++ $p<0.01$ compared to $t=0$ within the betamethasone group. (Wilcoxon signed-rank test).

a: elastase (IU/mg), **b:** thrombin (IU/mg), **c:** protein.

Discussion.

In the present study we demonstrated that preterm lambs born after antenatal administration of betamethasone 24 hours before delivery, had far more superior lung function as was indicated by lower oxygenation index and AaDO₂ and better dynamic compliance. As a consequence they needed less ventilatory support than controls. We demonstrated a decrease in PMNs and a rise in plasma beta-glucuronidase concentration in controls, which was attenuated in preterm lambs treated with antenatal betamethasone. The decrease in PMNs and the rise in beta-glucuronidase were considered to result from activation of an inflammatory reaction and might be the result of oxidative stress or barotrauma, which is considered to be injuring to the lung. However whatever the cause was, this effect was attenuated by antenatal betamethasone. Also activation of clotting was attenuated as was indicated by a lack of change in thrombin inhibition, no decrease in aPTT and a longer clot lysis time in the betamethasone treated lambs. Whether the attenuation of the inflammatory reaction and the activation of clotting was the direct result of antenatal glucocorticoids or the result of less severe RDS and less ventilatory support can not be concluded from this study.

Although animal studies have raised concern about the effects of antenatal glucocorticoids on the developing brain, no adverse effects have been demonstrated in humans from a single course of antenatal betamethasone. The incidence of intracerebral hemorrhage and periventricular leukomalacia even seems to be decreased.^[14] Antenatal administration of glucocorticoids (especially betamethasone) is associated with far less side effects than postnatal use of steroids (especially dexamethasone).

Therefore, the known beneficial effects of antenatal glucocorticoids on lung function of preterm infants after birth still justify its use.^[3,14] Also in preterm lamb studies improvements in compliance, ventilation efficiency and lung volumes and decreased protein permeability have been found after administration of betamethasone to the ewes.^[15,16]

Several mechanisms, including maturational effects, anti-inflammatory effects, effects on clotting and effects on lung water resorption, can explain the benefits of antenatal glucocorticoids.

Maturational effects of antenatal glucocorticoids include direct effects on lung cell maturation and differentiation and on remodeling of the alveolar architecture including thinning of the interstitial components of the lung. Glucocorticoids also up-regulate genes that are involved in the synthesis of surfactant proteins and phosphatidylcholines, resulting in stimulation of surfactant production. Further maturational effects of glucocorticoids include stimulation of antioxidant enzymes.^[4]

We assume that the anti-inflammatory effects of antenatal glucocorticoids also form part of the beneficial effects on lung function. We observed in the preterm lambs pre-treated with antenatal betamethasone higher numbers of PMNs than in controls. This finding is

in accordance with the known effect of glucocorticoids that they induce a release of leukocytes from the marginating pool. It is also known that glucocorticoids have anti-inflammatory properties in the lung itself. Glucocorticoids decrease neutrophil adherence to (lung) vascular endothelium, inhibit migration of neutrophils to inflammatory sites, inhibit antigen processing by macrophages, suppress T-helper cell function, inhibit the production of cellular mediators (prostaglandins, interleukins and other cytokines) and inhibit phagocytosis.^[4] Therefore the lack of change in PMNs can be explained as a decreased migration of PMNs to the interstitium of the lungs and the alveoli.^[17] Further support for the importance of the anti-inflammatory properties of antenatal glucocorticoids on postnatal lung function can be found from studies that have described the beneficial effects of early postnatal steroids on lung inflammation.^[7]

Attenuation of activation of clotting is another beneficial effect of antenatal glucocorticoids. The initial aPTT was longer in the betamethasone treated lambs than in the controls as a reflection of a more efficient down-regulation of the clotting system and not a reflection of clotting factor consumption at that moment. In contrast, the initial increase of aPTT in the controls reflects consumption of probably factor VIII in a state of activated clotting which is corrected in the following hours. The more effective inhibition of thrombin in the betamethasone treated lambs is in accordance with the observed higher aPTT. The reduced capacity for clotting in the betamethasone treated lambs is balanced with a lower fibrinolytic activity, which is probably due to reduced tissue plasminogen activator release as an effect of glucocorticoids.^[18,19]

A rather well known aspect of the working mechanism of antenatal glucocorticoids is the regulation of fluid balance in the lungs. In the presented study we demonstrated that the water content of parts of the left lung of betamethasone treated lambs was lower than the water content of parts of the left lung of controls. The difference was significant for the lower lobe but not for the upper lobe, but this may be due to the sample size. Although the lambs were ventilated in supine or lateral position and turned every 2 hours, one could assume that the lower, dependent parts of the lungs were ventilated and perfused more than the upper parts. The differences between the two groups would therefore be clearer in the lower parts of the lungs than in the upper parts. Glucocorticoids regulate fluid resorption from the lungs as well by decreasing pulmonary vascular resistance as by stimulation of the production of mRNA for lung epithelial sodium channels and aquaporin-1 and aquaporin-4 water channels, which play an important role in transporting water.^[4,6,20] The effect of glucocorticoids on edema formation has further been declared by direct vasoconstriction, decreased permeability of pulmonary capillaries for albumin and fluid without accompanying effect on albumin permeability of the systemic vasculature in lambs.^[15,16]

We assume that the attenuation of early inflammatory reaction and clotting in combination with the decreased water content of the lungs after antenatal glucocorticoid administration are additional factors explaining the beneficial effects of antenatal glucocorticoids on postnatal lung function and decreased need for ventilatory support.

Possibly this part of the working mechanism of antenatal glucocorticoid administration can help us to find future more specific therapeutic interventions for neonatal RDS.

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Early activation of systemic inflammation and development of neonatal RDS in preterm lambs.

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Abstract.

To investigate the relationship between early activation of the inflammatory reaction and the development of neonatal RDS in preterm lambs and the effects of endotracheal surfactant and antenatal glucocorticoids on this inflammatory reaction we studied preterm lambs.

Lambs were delivered by cesarean section at 132 days gestational age (term 145) and ventilated during 8 hours. Blood was analyzed for PMNs and β -glucuronidase as markers of systemic activation of the inflammatory reaction before, at 15 minutes after birth and 2 hourly afterwards. Bronchoalveolar lavage fluid was analyzed for elastase 2 hourly. We studied 9 lambs without therapeutic intervention, 8 lambs after surfactant (Alveofact[®] 50 mg/kg endotracheally immediately after delivery) and 9 lambs after antenatal glucocorticoid administration (betamethasone 0,5 mg/kg intramuscular 24 hours before the cesarean section).

We found systemic activation of the inflammatory reaction reflected by a decrease in PMNs and an increase in β -glucuronidase within 15 minutes after birth. Activation of the inflammatory reaction was not attenuated by surfactant administration, but it effectively was attenuated by betamethasone. We found neither a correlation between the systemic inflammatory reaction and clinical severity of neonatal RDS, nor between the inflammatory reaction and histologic severity of RDS in any of the groups.

We conclude that systemic activation of the inflammatory reaction within minutes after birth does not influence the development of neonatal RDS in the first 8 hours after delivery.

Introduction.

Neonatal Respiratory Distress Syndrome (neonatal RDS) is a disease in preterm infants characterized by surfactant deficiency and surfactant dysfunction in combination with structural immaturity of the lungs. There is increasing evidence that inflammation contributes to the development of this disease.

Murch et al. found excessive polymorphonuclear leukocytes (PMNs) and alveolar macrophages in the lungs of infants who died because of acute RDS.^[1] Other authors found PMNs, alveolar macrophages and pro-inflammatory cytokines in bronchoalveolar lavage fluid (BALF), reflecting local activation of inflammation in the lungs of preterm infants with RDS, prone to the development of bronchopulmonary dysplasia.^[2]

Not only activation of an inflammatory reaction in the lungs itself, but also systemic activation of the inflammatory reaction was found. Brus et al. demonstrated that preterm infants with RDS had lower PMN counts on day 2 than preterm infants without RDS and the severity of neutropenia was related with the severity of neonatal RDS and the degree of ventilatory support.^[3,4] Ferreira studied preterm infants with a birthweight of 500-1250 grams and found a relationship between the drop in PMNs within 2 hours after birth and the need for ventilation at the age of 12 hours and 7 days. The drop in PMNs is a manifestation of systemic activation of the inflammatory reaction with aggregation of PMNs and extravasation on the place of injury, in case the lungs.^[5-7] The lung vasculature contains a large reservoir of PMNs (the margined pool). In response to circulating complement factors or endotoxins and interleukins, secretory products of phagocytes and platelet-derived products, PMNs roll over and adhere to the endothelium. This is the first step towards migration of PMNs through the endothelium in the alveolar lumen.^[8] In lungs of patients dying of RDS, numerous activated PMNs can be found in alveolar spaces and PMNs are considered to play an important role in the inflammatory reaction in response to lung injury.^[8] In addition, Carlton et al. described that PMNs disappeared from blood due to sequestration in the lungs of preterm lambs with RDS, causing increased vascular permeability. This effect was attenuated in neutropenic lambs, indicating that PMNs contribute to the pathogenesis of RDS by increasing vascular permeability and promoting lung edema.^[9]

These studies indicate that systemic activation of the inflammatory reaction plays an important role in the development of RDS and in the development of bronchopulmonary dysplasia. However those studies were performed hours after birth and as Ferreira already noticed, the drop in PMNs that was found within 2 hours after birth was transient and PMNs increased 5-fold within the next 6 hours.^[6] Based on these studies, the relationship of early activation of the inflammatory reaction and the development of neonatal RDS, which still has to develop its full-blown picture at that moment, has not been fully elucidated.

We studied the relationship between early systemic activation of the inflammatory reaction and the development of neonatal RDS in a preterm lamb model. We also studied the histology of the lungs after 8 hours ventilation. Because of the beneficial effects of endotracheal

surfactant administration and of antenatal glucocorticoid administration on the development of RDS, we also studied the effects of those two therapies on the activation of the inflammatory reaction in relationship to the development of RDS.^[10,11]

Methods and materials.

The experiments were performed in the central animal laboratory of the University of Groningen, under approved animal care protocols of the University of Groningen with concern for animal welfare.

Treatment of the animals.

Before starting each single experiment we randomized by coin method for no treatment other than conventional ventilation (the NT group), surfactant administration and ventilation (surfactant group) or antenatal glucocorticoid administration and ventilation (betamethasone group). Ewes who were randomized for antenatal glucocorticoid administration were injected with 0,5 mg/kg betamethasone 24 hours before the planned cesarean section. The ewes were sedated with Sevoflurane[®] inhalation anesthesia and a cesarean section was performed under local anesthesia with subcutaneous prilocaine. Before clamping the cord, an umbilical arterial catheter was inserted, blood was sampled and the lambs were intubated orotracheally with a cuffed 5.5-mm ID tube and the airways were suctioned once. After birth the lambs were dried and weighed while 10 careful manual inflations with maximal peak inspiratory pressure of 20 cm H₂O and tidal volume of 5-8 ml/kg were given to promote aeration of the lungs. Pressure and tidal volume were measured using a Siemens air manometer (Siemens, Munich, Germany) and a Bior monitoring system (Bior monitoring systems, Irvine, California). If randomized to surfactant administration, surfactant 50 mg/kg (Alveofact[®], Boehringer Ingelheim, Germany), was administered endotracheally immediately after delivery, as a single bolus, followed by the manual inflations. Alveofact[®] is a bovine derived surfactant, containing 88% phospholipids.

All lambs were mechanically ventilated during 8 hours by conventional ventilation with a time-cycled pressure-limited infant ventilator (Babylog 8000, Dräger, Lübeck, Germany). Initial settings were frequency 60/min, inspiratory time and expiratory time 0.5 sec., gas flow 8 l/min, peak inspiratory pressure 20 cm H₂O, positive end-expiratory pressure 4 cm H₂O, FiO₂ 0.4. Settings were instantly adjusted to achieve tidal volumes of 5-7 ml/kg and an expiratory minute volume of about 300-350 ml/kg/min and to maintain transcutaneous O₂ saturation between 92 and 95% (measured with pulse oximeter Nellcor, Hayward, California, USA) and PaO₂ between 6.0 and 8.0 kPa. During the study, peak inspiratory pressure was adjusted to achieve PaCO₂ values of 5.5 to 7.0 kPa. The lambs were ventilated in lateral position and 2-

hourly, after endotracheal suctioning, they were placed on the contralateral side.

The umbilical arterial catheter was used for blood sampling and measurement of blood pressure. NaCl 0.9% was continuously infused on this catheter at a rate of 2.0 ml/h. During the study blood pressure was maintained in the normal range aiming at a mean blood pressure of 40 mmHg. A catheter for the administration of glucose 10% (3 ml/kg/h) and sodium pentobarbital, was placed in the vena cava inferior through the umbilical vein. Serum glucose levels were measured 2-hourly and were kept in the normal range (4-9 mmol/l) by adjusting the amount of glucose that was infused. Body temperature was measured continuously by a rectal thermometer and was kept at 38°C by using preheated mattress and blankets. Sodium pentobarbital (Nembutal®) was given for sedation, initially 15 mg/kg/h; dosage was adjusted according to activity, blood pressure and heart rate of the lamb.

Eight hours after delivery, the lambs were killed with an overdose sodium pentobarbital and a thoracotomy was performed to remove the lungs.

Blood sampling.

Heparinized blood samples of 0.4 ml were used to assess PaO₂, PaCO₂, and pH, using an ABL 330 bloodgas analyzer (Radiometer Co, Copenhagen, Denmark).

0.25 ml of each blood sample was anticoagulated with EDTA 0.01 M. This blood was used to prepare blood smears to determine by light microscopy total number of leukocytes and differential cell counts. The other blood was anticoagulated with citrate 0.31% and placed on ice. After centrifuging at 1100 g for 10 minutes, the plasma was stored at -80°C until β -glucuronidase was determined.

Bronchoalveolar lavage.

Immediately after intubation the airways were suctioned. Bronchoalveolar lavage was performed 2-hourly by injecting 1 ml/kg NaCl 0.9% intratracheally with immediate aspiration, which was repeated twice. The residual BALF was centrifuged at 1100 g and the supernatant was stored at -80°C until elastase concentration was determined.

Assays.

Severity of neonatal RDS was expressed as oxygenation index ($OI = \text{Mean airway pressure} \times \text{FiO}_2 / \text{PaO}_2$) and alveolar-arterial partial O₂ tension difference ($AaDO_2 = \text{FiO}_2 (P_{\text{atm}} - P_{\text{H}_2\text{O}}) - \text{PaCO}_2 - \text{PaO}_2$).

The total number of leukocytes was determined microscopically after gentian violet

staining of EDTA blood, using a counting chamber. Giemsa stained smears were used to determine differential cell counts making calculation of the number of PMNs possible.

β -glucuronidase is released from azurophilic granules from PMNs after activation.^[12] We measured β -glucuronidase in plasma instead of elastase (also released from azurophilic granules after activation of neutrophils) because elastase is inactivated in blood by α -1-antitrypsin immediately after release. β -glucuronidase was measured according to Baehner.^[13] Specificity of substrate used in the assay (p-nitrophenyl- β -D-glucuronid) was tested by adding a cocktail of inhibitors (C1esterase inhibitor, hirudine, α -1-antitrypsin and aprotinine), which inhibit complement, thrombin, trypsin, chymotrypsin, plasmin, kallikrein and activated protein C. In the presence of these inhibitors we found no reduction of β -glucuronidase activity, which led to the conclusion that the substrate was specifically converted by β -glucuronidase. β -glucuronidase was expressed in optical density (OD) corrected for the number of PMNs.

Elastase in BALF (a marker of local activation of PMNs in the lung) was measured by incubating BALF with elastase substrate (Omnilabo, Breda, The Netherlands) for three hours at 37°C. The recorded OD value at 405 nm using the microplate reader was compared to a standard porcine pancreatic elastase. Specificity of substrate used in the assay was tested by adding a cocktail of inhibitors (C1esterase inhibitor, hirudine and aprotinine), which inhibits complement, thrombin, chymotrypsin, plasmin, kallikrein and activated protein C, to the test substance. In the presence of this cocktail, we found no reduction of elastase activity, which led to the conclusion that the substrate was specifically converted by elastase.

To correct for dilution, values of elastase were corrected for the measured urea in BALF. Urea was determined according to Marsh et al.^[14]

Histology.

In each lamb, a part from the left upper and lower lobe of the lungs was removed and fixated in 10% buffered formalin suspension. After fixation the lung parts were processed, embedded in paraffin and stained with hematoxylin and eosin. Two independent investigators who were blinded for the treatment groups performed light microscopic evaluation on one representative slide of the left upper and lower lobe. We judged the inflation pattern and divided the patterns in homogenous inflation, irregular inflation (combination of good inflation (or hyperinflation) and atelectasis) or severe atelectasis. We also evaluated the degree of lung injury by using a quantitative scoring system modified from Doctor.^[15] We used 5 variables: atelectasis, inflammation, alveolar fibrin, hyaline membranes and alveolar hemorrhage. Each item could be scored 1: none, 2: minimal, 3: moderate and 4: severe. The minimal total score (no lung injury) therefore was 5, the maximal score (severe lung injury) was 20.

Separately we scored the presence of alveolar PMNs. We scored no PMNs, moderate PMNs or many PMNs.

Data and statistical analysis.

Oxygenation index, number of PMNs, β -glucuronidase and elastase in BALF data are presented as boxplots with median, quartiles, ranges and extremes. To compare the clinical and laboratory parameters between the groups, Mann Whitney tests were used because data were not normally distributed. Nominal variables (histologic pattern) are presented as frequencies of occurrence. To compare the groups, Kolmogorov Smirnov test was used. Tests were considered significant if $p < 0.05$.

For correlation analysis we used the mean oxygenation index and AaDO₂ over the 8-hour period for each lamb as measurements of mean severity of neonatal RDS. We used the number of PMNs, β -glucuronidase and elastase in BALF at $t = 8$ hours after birth and the lowest number of PMNs, the highest β -glucuronidase and the highest elastase in BALF from each lamb as measurements of activation of the inflammatory reaction. We correlated severity of neonatal RDS to measurements of the inflammatory reaction, severity of neonatal RDS to histologic findings and measurements of the inflammatory reaction to histologic findings. Spearman correlation coefficients were determined. Correlations were considered significant at the 0.05 level (2-tailed).

Results.

We studied 26 preterm lambs of 132 days gestational age (term 145 days), 9 without therapeutic intervention (NT lambs), 8 after surfactant administration and 9 after antenatal betamethasone administration.

During the study period oxygenation index (figure 1) as well as AaDO₂ (data not shown) decreased in all groups. No statistically significant differences were found between the 3 groups.

Figure 1.

Oxygenation index of NT group (white), surfactant group (gray) and betamethasone group (black), at 15, 120, 240, 360 and 480 minutes after birth. Data are presented as median, quartiles, ranges and extremes (+).

The figures 2a and 2b show number of PMNs and β -glucuronidase (corrected for number of PMNs) in plasma.

PMNs (figure 2a) decreased in the first 15 minutes after birth in the NT group and in the surfactant group. During the following 8 hours of ventilation a gradual increase of PMNs in plasma was demonstrated in both groups. Between the 2 groups no statistically significant differences were found. In the betamethasone group no change in number of PMNs could be demonstrated. PMNs were significantly higher during the entire study period in the betamethasone lambs than in the other two groups.

β -glucuronidase (figure 2b) increased in the first 15 minutes after birth in the NT group and in the surfactant group and normalized afterwards. Between the 2 groups no statistically significant differences were found. In the betamethasone group no increase in level of β -glucuronidase could be demonstrated. β -glucuronidase was significantly lower in the betamethasone lambs than in the other two groups in the first 4 hours after birth.

Figure 2.

Measurements of inflammatory parameters in plasma of NT group (white), surfactant group (gray) and betamethasone group (black), at $t=0$ (just before birth) and 15, 120, 240, 360 and 480 minutes after birth. Data are presented as median, quartiles, ranges and extremes (+).

a: Number of polymorphonuclear leukocytes (PMNs, number/ml), **b:** β -glucuronidase (corrected for number of PMNs, OD/PMN).

* $p<0.05$ and ** $p<0.01$ betamethasone group versus NT group and surfactant group.

Elastase in BALF (figure 3) increased significantly in the first 2 hours after birth in all three groups. Elastase in BALF of surfactant lambs was significantly higher than in BALF of the other 2 groups during the entire study period. Remarkably in these results are the large ranges of measured values within the groups of lambs.

Figure 3.

Concentrations of elastase in bronchoalveolar lavage fluid corrected for urea concentrations of NT group (white), surfactant group (gray) and betamethasone group (black), at $t=0$ (just before birth and after intubation), 120, 240, 360 and 480 minutes after birth. Data are presented as median, quartiles, ranges and extremes (+).

$p<0.01$ for surfactant group versus NT group and betamethasone group.

Results of histologic examinations were the same for the upper lobe and the lower lobe. The results of the lower lobe are given in figure 4-6.

Figure 4.

Absolute frequencies of distribution of pattern at histologic examination of the left lower lobe for NT group (white), surfactant group (gray) and betamethasone group (black).

Figure 5.

Lung injury score of the left lower lobe for NT group (white), surfactant group (gray) and betamethasone group (black).

Figure 6.

Scoring of number of PMNs at histologic examination of the left lower lobe for NT group (white), surfactant group (gray) and betamethasone group (black).

Between the 3 groups no statistically significant differences were found concerning pattern of inflation. In none of the betamethasone lambs severe atelectasis was found, but in general the lungs of betamethasone lambs showed the same histologic pattern as the other groups. Also the lung injury score showed the same distribution in the 3 groups. Scores of all lambs varied between 5 and 15, no statistically significant differences were found. In 2 of 9 NT lambs, PMNs were found at histologic examination. In 3 of 8 surfactant lambs PMNs were found at histologic examination, whereas in none of the lambs treated with antenatal betamethasone, PMNs were found at histologic examinations.

To study a correlation between severity of RDS and severity of activation of the inflammatory reaction, we correlated mean oxygenation index and mean AaDO₂ to PMNs, β -glucuronidase and elastase in BALF. We found neither correlations for all lambs together, nor for the separate groups.

To study a correlation between severity of RDS and histologic pattern, we correlated mean oxygenation index and mean AaDO₂ to scoring of pattern, lung injury score and PMNs at histologic examination. We found neither correlations for all lambs together, nor for the separate groups.

To study a correlation between severity of inflammatory reaction and histologic pattern, we correlated PMNs, β -glucuronidase and elastase in BALF to scoring of pattern, lung injury score and PMNs at histologic examination. We found neither correlations for all lambs together, nor for the separate groups.

Discussion.

We studied the relationship between early activation of the inflammatory reaction and the development of neonatal RDS and the effects of surfactant and antenatal betamethasone. We

found systemic activation of the inflammatory reaction reflected by a decrease in PMNs and an increase in β -glucuronidase within 15 minutes after birth in preterm lambs ventilated for RDS, with a recovery afterwards. This is in agreement with our previous study.^[16] The activation of the inflammatory reaction was not attenuated by surfactant administration, but antenatal betamethasone administration effectively attenuated this activation. However, we found neither a correlation between the systemic activation of the inflammatory reaction and clinical severity of neonatal RDS, nor between the inflammatory reaction and histologic findings. Although antenatal betamethasone attenuated the inflammatory reaction and no PMNs were found in the lungs of the lambs, neither the clinical severity of neonatal RDS, or histologic findings were different in the betamethasone group compared to the NT and surfactant group. Therefore, we conclude that activation of the inflammatory reaction within minutes after birth does not influence the development of neonatal RDS in the first 8 hours of life.

We were surprised to find no relationship between the early systemic activation of the inflammatory reaction and the development of RDS. One could argue that the lambs in our model (132 days gestational age) were too old to suffer from severe RDS. However, in a pilot study we observed that lambs of 132 days gestational that were not ventilated, died with the clinical picture of neonatal RDS: tachypnea, dyspnea, grunting, cyanosis, respiratory acidosis and ultimately respiratory failure. Ventilation of lambs younger than 132 days gestational age is not always possible without surfactant.^[17] Therefore we chose lambs of 132 days gestational age and we assumed that RDS in these animals was severe enough to be representative for infant RDS.

Our finding is in contrast to previous studies in humans, as well as in lambs. Ferreira et al. described a strong the relationship between neutropenia within 2 hours after birth in infants between 500 and 1250 grams, and severity of RDS during the first postnatal week.^[6] However this study probably merely investigated the relationship between neutropenia and pulmonary problems after the first day of life and not the development of RDS within 8 hours after birth. Brus et al. demonstrated in preterm infants between 27 and 33 weeks gestational age that decreased PMN counts 2 days after birth were correlated with increased RDS severity.^[4] In this study the inflammatory reaction was demonstrated 2 days after birth and not within minutes after birth as in our study.

It is possible that in previous animal and human studies other mechanisms than RDS, for example hyperoxia, hypoxia, acidosis and tissue injury, caused the inflammatory reaction. In our study oxygen administration was carefully monitored, avoiding hyperoxia or hypoxia. In contrast to other investigators, we used no transcutaneous intravenous access or tracheotomy, which are considered important activators of an inflammatory reaction.^[18-20] Carlton et al. studied preterm lambs of 127 days gestational age: they found that PMNs disappeared from blood within 2 hours after birth due to sequestration in the lungs causing increased vascular permeability, which seemed to be related to the severity of RDS.^[9]

However these lambs were surgically prepared in utero and had catheters placed in veins and lymph vessels. It is possible that the activation of the inflammatory reaction and the

accumulation of neutrophils in the lungs was not related to RDS but merely to surgical preparation and the presence of catheters.

In our study no attenuating effect of surfactant administration on the early activation of the inflammatory reaction was demonstrated. The immediate clinical effect of surfactant administration is improved oxygenation by an increase in functional residual capacity and by a decrease in ventilation-perfusion mismatch. As a result, ventilatory support can be decreased.^[10,21] Apart from the immediate “mechanical” effect of surfactant on functional residual capacity, surfactant is considered to have immunomodulatory properties. Surfactant proteins enhance chemotaxis, enhance phagocytosis, enhance killing of some bacteria, suppress the production of free radicals and cytokines, stimulate immunoglobulin production and stimulate lymphocyte proliferation.^[22] Surfactant lipids enhance phagocytosis and killing, suppress the production of free radicals and inhibit lymphocyte proliferation.^[22] Therefore surfactant administration can be expected to have an attenuating effect on an existing inflammatory reaction. The systemic inflammatory reaction we demonstrated was so short after birth that the anti-inflammatory effects of surfactant probably could not yet be found.

Surprisingly, we found higher elastase levels in BALF after surfactant administration. We evaluated our assay, but found no contamination. Analysis of surfactant revealed no elastase in the surfactant vial itself. Usually, elastase in BALF, which is released from activated PMNs, is considered as a good marker for inflammation locally in the lungs.^[23] Merritt et al. demonstrated that elastase levels were increased in 3 days old preterm infants with RDS eventually developing bronchopulmonary dysplasia.^[24] Higher elastase levels seem harmful because elastase is a proteinase that may attack host proteins as elastin and fibronectin, thereby changing the normal surrounding tissue and the inflammatory process.^[25] Elastase is released from activated PMNs; therefore the higher elastase levels in surfactant treated lambs suggest more local inflammation in the alveoli after surfactant administration. We could not confirm this by histologic examination. We therefore think that after surfactant administration, sampling of BALF is performed from a greater part of the lungs due to increased functional residual volume, than in the other groups, leading to higher elastase levels in the assay. This finding impedes us to draw a conclusion about the local anti-inflammatory effect of surfactant.

Although in our study, antenatal administration of glucocorticoids attenuated the activation of the inflammatory reaction, no improvement of the clinical and histologic evidence of RDS could be demonstrated. This supports our conclusion that the early activation of the inflammatory reaction is not related to the development of RDS.^[26] The benefits of antenatal glucocorticoids on postnatal lung function can be explained by their maturational effects and effects on water clearance in the lungs.^[27-29] Anti-inflammatory properties do not explain the beneficial effects of antenatal betamethasone.

We conclude that systemic activation of the inflammatory reaction immediately after birth is neither related to development of neonatal RDS as evidenced by the need for ventilatory support nor to the histologic findings. Systemic activation of the inflammatory reaction is attenuated by antenatal glucocorticoid administration and not by surfactant administration. This

further supports the conclusion that the activation of the inflammatory reaction immediately after birth in the preterm lambs is not related to the development of neonatal RDS.

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Chapter 8

General discussion.

General discussion.

Neonatal Respiratory Distress Syndrome (neonatal RDS) is a disease of preterm infants. Structural immaturity of the lungs and immaturity of the pulmonary surfactant metabolism, resulting in surfactant deficiency and surfactant dysfunction are known causes of neonatal RDS. Apart from these factors there is increasing evidence that activation of inflammation and clotting contributes to the progression of this disease. However most authors describe abnormalities in broncho-alveolar lavage fluid and plasma that can be found on various days after birth, probably more reflecting the role of the inflammatory reaction in the progression from neonatal RDS to bronchopulmonary dysplasia (BPD) than the role of inflammation in neonatal RDS itself.^[1]

The aim of this thesis is to explore the role of activation of inflammation and clotting in the development and the severity of neonatal RDS. In preterm lambs we studied the exact moment of activation of the inflammatory reaction and the relationship between the activation of the inflammatory reaction and the severity of the respiratory problems. We also compared the impact of different ventilatory modes (conventional ventilation and high frequency oscillatory ventilation on activation of the inflammatory reaction. In addition we studied the effects of already established therapies as endotracheal surfactant administration and antenatal glucocorticoid administration on activation of the inflammatory reaction with respect to the development of respiratory distress.

We describe that in preterm ventilated lambs with neonatal RDS, the inflammatory reaction is activated immediately after delivery. Also in healthy term lambs an inflammatory reaction is demonstrated immediately after delivery, but this reaction is transient and less pronounced than in preterm ventilated lambs. We assume that term lambs have an adequate counter-regulation to stop the inflammatory reaction in the absence of a concurrently ongoing injurious stimulus (such as ventilation or immature lungs with RDS). We do not demonstrate a significant correlation between the activation of the inflammatory reaction in the preterm lambs and the development of RDS or the needed ventilatory support. After antenatal glucocorticoid administration, no signs of activation of the inflammatory reaction can be found. However, antenatal glucocorticoids do not alter the histologic findings of the lungs of the preterm ventilated lambs. This further supports the finding that the inflammatory reaction is not related to the severity of RDS. Surfactant administration does not attenuate the activation of the inflammatory reaction, nor the development of RDS.

These findings are in contrast with previous studies demonstrating a relationship between the severity of activation of inflammation and the severity of neonatal RDS.^[2-4] Previous studies were performed in animals or humans that were possibly asphyxiated. Hypoxia is a well-known trigger for the initiation of the inflammatory reaction.^[5-7] Other authors used venapuncture for blood sampling and tracheotomy in animal studies for intubation. Skin incision is also well known for initiating an inflammatory reaction.^[8-10]

Perhaps the development of RDS in those previous studies was not causally related to activation of the inflammatory reaction but only accompanied by activation of the inflammatory reaction by another mechanism.

Our findings are in accordance with studies that demonstrated that chronic inflammation of fetal membranes and amniotic fluid, which accompanies preterm birth often, is associated with a decrease in severity of RDS.^[11] Pro-inflammatory cytokines in the amniotic fluid seem to induce lung maturation.^[12,13] An increased amount of surfactant proteins is found after direct intra-amniotic interleukin-1 (IL-1) administration and after intra-amniotic endotoxin administration, which induces cytokine production.^[14,15] Therefore the inflammatory reaction we demonstrate immediately after delivery can indeed be beneficial in lowering the risk of development or can attenuate the severity of neonatal RDS.

However, persistence of the inflammatory reaction can induce lung injury. Overexpression of pro-inflammatory cytokines (tumor necrosis factor α , IL-1, IL-6 etc.), that are released by activated polymorphonuclear leukocytes (PMNs), can interfere with postnatal alveolarization.^[11] The development of bronchopulmonary dysplasia is associated with the presence and persistence of pro-inflammatory cytokines, chemokines and other inflammatory products (for example oxygen radicals).^[1,16-19]

An inflammatory reaction is a response to cellular injury that serves as a mechanism initiating the elimination of the noxious agent and damaged tissue. One can imagine that birth is a process that is potentially damaging to the organism. It can be assumed that birth itself initiates an inflammatory reaction. In term birth this process is strictly regulated. Whenever there is no reason to continue the inflammatory reaction, counter-regulatory mechanisms inhibit the reaction itself. PMNs are preprogrammed for apoptosis after migration in the tissues unless they are instructed otherwise.^[20] Normally, PMN migration across the lung microvasculature does not cause a major change in epithelial permeability.^[21] However if the circulating PMNs have been exposed to inflammatory stimuli (for example intrauterine inflammation), or whenever the injuring stimuli persist (for example ventilation after delivery), the already “primed” PMNs can react and can cause increased permeability after they have undergone the complete process of rolling, adherence and migration.

Preterm birth can be the result of an inflammatory reaction on microorganisms or of a chronic inflammatory reaction of unknown origin. After delivery, several potential PMN activating processes are initiated, for example: artificial ventilation, hypoxia, hyperoxia, venous accesses, penetration of skin barriers with the risk of introduction of microorganisms, infection. Complicating factor for preterm infants or lambs, compared to term delivered infants or lambs, is insufficient counter-regulation of an inflammatory reaction. Pulmonary cells of preterm infants may be unable to down-regulate inflammation because of low expression of anti-inflammatory cytokines as IL-10 or possibly cytokine antagonists.^[22] In preterm infants, preprogramming of PMNs for apoptotic changes seemed to be delayed.^[23]

If the infant is able to inhibit and stop the acute inflammatory reaction, no symptoms of RDS or BPD will develop. However, whenever a new inflammatory stimulus triggers the

already primed PMNs the inflammatory reaction will persist or will be activated acutely. The symptoms of this acute inflammatory reaction will be dependent on the site of injury. Systemic manifestations as in sepsis or sepsis like disease with circulatory insufficiency can be expected in case of blood stream infections with microorganisms. Respiratory problems can be expected in case of local inflammatory reaction after ventilation with high inspiratory oxygen concentrations. Necrotizing enterocolitis can be expected after ischemia or distension of the gastrointestinal tract.

If the inflammatory reaction persists or is reactivated locally in the lungs and anti-inflammatory mechanisms are insufficient, the inflammatory reaction can be injuring itself, leading to BPD.

For the future it is important to prevent first and/or second hits to activate PMNs. Studies are needed regarding investigation of the unknown inflammatory reaction that can initiate preterm delivery.^[24] If possible, the intrauterine inflammation has to be prevented. Maybe that is the way to prevent preterm birth and to prevent BPD.

Anti-inflammatory medications given before delivery, like antenatal glucocorticoids can prevent the initiation of the inflammatory reaction. Antenatal glucocorticoids impede the activation of the inflammatory reaction, but the beneficial effect regarding the development of BPD can be questioned. If there is already an inflammatory reaction, antenatal glucocorticoids possibly have a negative effect because they interfere with alveolarization, which may lead to BPD.^[25] Therefore, future studies have to be directed at the development of anti-inflammatory drugs that do not interfere with lung development on the long term. Possibly there is a role for non-steroidal anti-inflammatory drugs like Ibuprofen or Indomethacin.^[26]

If preterm delivery, with or without an already activated inflammatory reaction, is inevitable we have to protect the newborn. Artificial ventilation is a trigger for continuation of the inflammatory reaction. Optimizing prenatal care possibly can abolish the need for artificial ventilation and gentle ventilatory strategies can possibly prevent the continuation of the inflammatory reaction. When there is surfactant deficiency, surfactant has to be administered to prevent further injury. However, an optimal way of administration with uniform distribution still has to be developed. Hyperoxia is a trigger for inflammation because oxygen radicals can be produced which are possible inflammatory mediators.^[27] Hyperoxia can be avoided if we know when the infant suffers from hyperoxia. So normal pO_2 values have to be determined for preterm newborns and until these are available, we possibly have to accept values as in utero instead of adult values. Other inflammatory triggers are infections or transcuteaneous access. General neonatal intensive care has to be optimized to prevent these triggers.

Aiming at the counter-regulatory side of the inflammatory coin is another option. Research in neonatology has to be aimed at the counter-regulatory mechanisms or at the balance between pro- and anti-inflammatory mechanisms. Possibly, up-regulating the anti-inflammatory mechanisms for example by IL-10 is a way to help the infant down-regulating

an inflammatory reaction.^[28,29] IL-10 is a potent anti-inflammatory cytokine. It suppresses the production of pro-inflammatory cytokines, it induces the production of anti-inflammatory molecules as IL-1- receptor antagonist, it inhibits the generation of oxygen radicals and the expression of adhesion molecules and it deactivates neutrophils and monocytes directly. IL-10 therefore seems a promising candidate for down-regulation of the inflammatory reaction.

New anti-inflammatory strategies can be directed at blocking the production of multiple pro-inflammatory cytokines simultaneously. Studies can be directed at the NF- κ B pathway and specific NF- κ B inhibitors are already implemented successfully in inflammatory arthritis.^[29] Maybe in the future NF- κ B inhibitors will be used for inhibition of the inflammatory reaction before or after delivery.

However, finding and influencing the balance between overzealous production of pro-inflammatory cytokines causing excessive tissue injury and overzealous production of anti-inflammatory cytokines leading to infections remains delicate. Maybe in the future the exact knowledge of the working mechanism of the inflammatory reaction and its antagonists will lead us the way to a safe intervention to protect the infant against lung injury.^[30]

Whatever the mechanism, it seems more and more important that treatment of preterm infants has to be individualized. When an inflammatory reaction has started, further stimulation of the inflammatory reaction has to be prevented and the counter-regulation has to be stimulated. When there is real injury or when there are invading microorganisms, the inflammatory response possibly has to be stimulated instead of inhibited to prevent ongoing damage. It is a challenge to give each individual patient the best treatment on the right moment.

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Chapter 9

Summary.

Summary.

Introduction.

Neonatal Respiratory Distress Syndrome (neonatal RDS) occurs in preterm infants, due to structural immaturity of the lungs and immaturity of the pulmonary surfactant metabolism, resulting in surfactant deficiency and surfactant dysfunction. Apart from these factors there is increasing evidence that inflammation and clotting contributed to the progression of this disease.

However most authors describe abnormalities in broncho-alveolar lavage fluid and plasma that can be found on various days after birth, probably more reflecting the role of the inflammatory reaction in the progression from neonatal RDS to bronchopulmonary dysplasia than the role of inflammation in neonatal RDS itself.

The aim of this thesis was to elucidate the role of local as well as systemic activation of the inflammatory reaction and clotting in the development and the severity of neonatal RDS in the period immediately after delivery. We intended to develop an animal model to investigate these systems and their mutual interactions. In this model we planned to compare the impact of different ventilatory modes (conventional ventilation and high frequency oscillatory ventilation) and of already established therapies as endotracheal surfactant administration and antenatal glucocorticoid administration on activation of the inflammatory reaction.

Summary of the methods and results.

We studied the activation of local as well as systemic inflammation and clotting in lambs suffering from neonatal RDS in the period between 0 minutes and 8 hours after preterm delivery.

We therefore studied preterm lambs, delivered by cesarean section after 132 days gestational age (term is 140-145 days). We sampled blood for analysis from an arterial umbilical catheter. We intubated the lambs orotracheally and ventilated them for 8 hours.

We analyzed blood for systemic activation of the inflammatory reaction (AP50 [complement system], polymorphonuclear leukocytes [PMNs] and β -glucuronidase [released from activated PMNs]) and for measurements of systemic activation of clotting (activated partial thromoplastin time [aPTT] and thrombin inhibition [as a modified measurement of antithrombin III]). We analyzed broncho-alveolar lavage fluid for measurements of local activation of inflammation (elastase [released from activated PMNs] and protein) and for measurement of local activation of clotting (thrombin).

We found that ventilated preterm lambs were suitable to investigate the activation of the inflammatory reaction and clotting, systemically as well as locally in the lungs. Without ventilation the lambs died of severe respiratory failure, indicating that we had chosen the right gestational age for our purpose. It was possible to ventilate the lambs for 8 hours

without (other) therapeutic interventions, such as surfactant administration and inotropic agents.

We found that signs of activation of the inflammatory reaction could be found already at 15 minutes after delivery. Systemic signs of the inflammatory reaction, especially activation of PMNs with release of β -glucuronidase, preceded local activation of inflammation in the lungs of ventilated preterm lambs with RDS. Afterwards, or due to activation of the inflammatory reaction, clotting was activated systemically as well as locally (*chapter 4*). Between conventional ventilation and high frequency ventilation no differences were found with respect to the severity of neonatal RDS. Similarly, the mode of ventilation did not effect the severity of activation of the inflammatory reaction and clotting.

We found that activation of the inflammatory reaction could be found immediately after delivery in preterm lambs as well as in spontaneously born non-ventilated term lambs. However the inflammatory reaction was less pronounced and lasted shorter in the term lambs than in the ventilated preterm lambs born by cesarean section (*chapter 5*). We therefore concluded that the activation of the inflammatory reaction that we demonstrated in preterm as well as term lambs probably was a normal physiologic response to birth. The degree of activation of the inflammatory reaction and the eventual persistence of the inflammatory reaction could be dependent of preterm delivery or ventilation or severity of respiratory problems.

Antenatal glucocorticoid administration improved postnatal lung function in the preterm lambs. Antenatal glucocorticoids attenuated early systemic activation of the inflammatory reaction and clotting significantly and decreased the water content of the lungs (*chapter 6*). We concluded that antenatal glucocorticoids had beneficial effects on the severity of neonatal RDS. However, it is already known that antenatal glucocorticoids induce structural maturation of the lungs and improve water clearance, which improves lung function. Whether the anti-inflammatory properties of antenatal glucocorticoids had an additional effect remained unclear.

We therefore studied the lungs by light microscopy after 8 hours of ventilation and found no improvement of lung histology, which would be reflected by lower lung injury scores or less atelectasis after antenatal glucocorticoid administration, despite of the absolute absence of PMNs after antenatal glucocorticoid administration (*chapter 7*). No correlation was found between clinical severity of neonatal RDS and severity of RDS in histologic examination. Therefore we concluded that antenatal glucocorticoids improved the clinical findings in RDS not by influencing the inflammatory reaction, but by accelerated lung maturation and increased water clearance.

Surfactant, administered immediately after delivery, did not reduce the need for ventilatory support and did not alter histologic findings of RDS. In addition we could not demonstrate an influence of surfactant administration on the activation of the inflammatory reaction (*chapter 7*). This finding was in accordance with the findings we described above, making us to conclude that in our studies the development of RDS was not caused by the activation of the inflammatory reaction immediately after delivery.

Conclusions.

From these studies we conclude in general that in preterm ventilated lambs with neonatal RDS, the inflammatory reaction is activated immediately after delivery. Clotting is activated later than the inflammatory reaction. Activation of the inflammatory reaction is not related to the development of neonatal RDS or the ventilatory support needed. After antenatal glucocorticoid administration, no signs of activation of the inflammatory reaction or clotting can be found, but histologic findings do not improve. This further supports the finding that the inflammatory reaction is not related to the development of RDS. Surfactant administration does not attenuate the activation of the inflammatory reaction.

The conclusion of this thesis is that activation of the inflammatory reaction immediately after birth is not related to the development of RDS during the first 8 hours after birth. Because of the signs of activation of the inflammatory reaction in term healthy lambs, we merely consider the activation of the inflammatory reaction immediately after delivery as a normal defensive mechanism, which is not related to respiratory problems but occurs after each delivery, possibly especially preterm delivery.

We can not deny the possibility that the inflammatory reaction contributes to the development of chronic lung disease. In the presence of a “second hit”, for example infection, ventilation on an immature lung, skin incision, or hypoxia, the inflammatory reaction will persist and/or become a chronic reaction. We assume that merely the persistence of the inflammatory reaction will be associated with the development of chronic respiratory problems or chronic lung disease than the acute reaction itself.

In the future are needed investigating the relationship between inflammation and respiratory problems with respect to the duration of the period that the inflammatory reaction persists. We assume that short-term activation is not related to respiratory problems, whereas long-term activation will be, especially to the development of bronchopulmonary dysplasia or chronic lung disease.

Future interventions aiming at prevention of respiratory distress and especially prevention of chronic lung disease possibly have to be aimed at prevention of the chronic inflammatory reaction by preventing “second hits”, for example hypoxia or hyperoxia, or infections. Possibly the use of anti-inflammatory drugs immediately before delivery and for the first days after delivery during periods of triggers of activation of inflammation can be advised.

Abbreviations.

AaDO₂	alveolar-arterial oxygen difference
APTT	activated partial thromboplastin time
ATIII	antithrombin III
BALF	bronchoalveolar lavage fluid
BPD	bronchopulmonary dysplasia
CARS	compensatory anti-inflammatory response syndrome
CPAP	continuous positive airway pressure
CV	conventional ventilation
FiO₂	fractional concentration of inspired oxygen
G-CSF	granulocyte colony stimulating factor
GM-CSF	granulocyte macrophage colony stimulating factor
HFFI	high frequency flow interruption
HFOV	high frequency oscillatory ventilation
HFV	high frequency ventilation
IL	interleukin
MIF	macrophage migrating inhibiting factor
NF-κB/IKK	nuclear factor κB/ inhibitor of κB kinase
NT	no treatment other than conventional ventilation
OI	oxygenation index
OD	optical density
PaCO₂	partial pressure of arterial CO ₂
PaO₂	partial pressure of arterial O ₂
PLA₂	phospholipase A ₂
PMNs	polymorphonuclear leukocytes
RDS	respiratory distress syndrome
SIRS	systemic inflammatory response syndrome
TNF	tumor necrosis factor
VEGF	vascular endothelial growth factor

Tenslotte.....

Over de overeenkomsten en verschillen tussen het lopen van een marathon en promoveren zijn al vele stellingen verschenen. Ik begrijp waarom.

Ik ben begonnen met hardlopen zonder van plan te zijn een marathon te lopen. Het werd steeds leuker en het ging steeds beter. Hardlopers met wie ik trainde hadden marathons gelopen en zeiden dat het lopen van een marathon voor elke hardloper het uiteindelijke doel is. Ze zeiden ook dat ik het ook zou kunnen. Ik twijfelde, want ik vond het niet zo nodig voor mijzelf, maar ja, als een ander het zegt, dan zal het wel zo zijn. Ik besloot het maar te proberen, maar de goede voorbereiding en de echte overtuiging dat het zou gaan lukken, ontbraken. Ik kreeg blessures en leek bevestigd te worden in mijn overtuiging dat het niks voor mij was.

Toch begon het steeds meer te kriebelen. Ik besloot dat ik een marathon wilde gaan lopen en zette me er nu voor de volle 100% voor in. Dat maakte dat het beter ging. Met leuke trainingsmaatjes doorliep ik de voorbereidingsfase. Zij hielpen als het zweten was als er getraind moest worden bij 25 °C (of meer), zij hielpen om door te zetten als de puf eruit was na 10 km of als er helemaal geen puf was om te trainen. Zij zeiden dat het niet erg was om langzaam te lopen, omdat dat nodig was om tot goede resultaten te komen. Zij waren er ook om samen blij te zijn als er weer een mijlpaal was bereikt.

Gelukkig waren er ook mensen die zeiden dat het helemaal niet nodig was zoveel te trainen. Zij zeiden dat ik ook voldoende tijd over moest houden voor andere dingen. Zij hielpen relativeren als het even helemaal niet ging.

Langzaam maar zeker naderde de dag waarop het allemaal zou moeten gebeuren.

De marathon zelf begon ik optimistisch: er leek tijd genoeg te zijn, het was zeker dat ik het zou halen, maar ja de manier waarop was nog wel een vraag. Het begon rustig, maar na 15 km had ik al zere benen en dacht ik dat het toch mis zou gaan. Alleen maar dankzij de hulp van de medelopers ging ik door. Uitstappen kon niet: het was mijn eer te na en ik kon het niet maken tegenover die mensen die vertrouwen in me hadden en me steeds geholpen hadden. Dus tanden op elkaar en doorzetten.

Toen de finish in zicht kwam, kwam ook het voldane gevoel. Onder het finish doek doorlopen is geweldig.

Daarom: trainingsmaatjes, medefinishers, supporters, thuisfront, vrienden, collega's, maar ook vrijwilligers en andere onzichtbare helpers: bedankt!

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Anneke