Chapter 4

Pentoxifylline reduces fibrin deposition and prolongs survival in neonatal hyperoxic lung injury
**Pentoxifylline reduces fibrin deposition and prolongs survival in neonatal hyperoxic lung injury**

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**ABSTRACT**

Bronchopulmonary dysplasia is a leading cause of mortality and morbidity in preterm infants despite improved treatment modalities. Pentoxifylline, a phosphodiesterase inhibitor, inhibits multiple processes leading to neonatal hyperoxic lung injury, including inflammation, coagulation and edema. Using a preterm rat model, we investigated the effects of pentoxifylline on hyperoxia-induced lung injury and survival. Preterm rat pups were exposed to 100% oxygen and injected subcutaneously with 0.9% saline or pentoxifylline 75 mg/kg twice a day. On day 10, lung tissue was harvested for histology, fibrin deposition, and mRNA expression, and bronchoalveolar lavage fluid was collected for total protein concentration. Pentoxifylline treatment increased mean survival by 3 days ($p = 0.0018$) and reduced fibrin deposition by 66% ($p < 0.001$) in lung homogenates compared with untreated hyperoxia-exposed controls. Monocyte chemoattractant protein-1 expression in lung homogenates was decreased, but the expressions of TNF-α, IL-6, matrix metalloproteinase-12, tissue factor, and plasminogen activator inhibitor-1 were similar in both groups. Total protein concentration in bronchoalveolar lavage fluid was decreased by 33% ($p = 0.029$) in the pentoxifylline group. Pentoxifylline treatment attenuates alveolar fibrin deposition and prolongs survival in preterm rat pups with neonatal hyperoxic lung injury, probably by reducing capillary-alveolar protein leakage.

**Key words:** hyperoxia, inflammation, coagulation, newborn rat, bronchopulmonary dysplasia.
INTRODUCTION

Bronchopulmonary dysplasia (BPD) is a leading cause of mortality and morbidity in preterm newborn infants with respiratory distress syndrome despite improved treatment modalities (22). Nowadays, BPD, defined by oxygen requirement at 36 wk of gestation, affects especially newborn infants born at <30 wk of gestation with a birth weight <1,200 g (1, 13). BPD is a multifactorial disease, characterized by decreased alveolarization and abnormal vascularization and associated with oxidative stress, barotrauma, surfactant deficiency, inflammation, alveolar fibrin deposition, nutrition, and genetic background (13-15). The (im)balance between initiating factors and host characteristics probably determines whether BPD will actually occur in the preterm infant.

The inflammatory response and coagulation cascade play important roles in the pathophysiology of acute and chronic lung disease in newborn infants and animal models. Increased levels of proinflammatory cytokines (TNF-α and IL-6) have been observed in tracheal aspirates of infants developing BPD (16-18). In addition, intra-alveolar and intravascular fibrin deposition has been detected in acute lung injury (2, 10, 11). In animal models, including premature baboons and neonatal mice and rats, exposure to hyperoxia results in chronic lung disease closely resembling BPD in preterm infants (3, 5, 8, 28, 37, 39).

In a previous study (37), our group demonstrated that genes involved in inflammation, coagulation, fibrinolysis, fibrosis, extracellular matrix turnover, alveolar development, edema, cell cycle, and oxidative stress response are differentially expressed in preterm rats with BPD secondary to exposure to hyperoxia (37). Expression profiles were in line with histopathology. The importance of extravascular fibrin deposition and upregulation of the coagulation cascade and inflammatory response in this model suggests a potential role for compounds with anti-inflammatory and anticoagulant properties. The methylxantine derivative pentoxifylline (PTX) has been used in the treatment of peripheral arterial diseases because of its positive effects on the capillary blood flow by reducing blood viscosity and improving erythrocyte deformability (27, 38). Moreover, PTX, a phosphodiesterase inhibitor, has significant anti-inflammatory effects, resulting in accumulation of intracellular cAMP. PTX decreases neutrophil sequestration, prevents the pulmonary vascular permeability to protein (12, 41, 43), and inhibits the production of free oxygen radicals (33). The inhibitory effect of PTX on proinflammatory responses and diffuse intravascular coagulation was demonstrated in baboons with lipopolysaccharide-induced endotoxemia (23). PTX was also effective in a radiation-induced lung model in mice by inhibiting TNF-α mRNA and protein production in lung tissue (29). Modulatory effects of PTX on the production of IL-6 were demonstrated in fetal rat type II pneumocytes exposed to hyperoxia and nitric oxide (6).

Because PTX inhibits multiple processes that contribute to the development of BPD, including inflammation, coagulation, and edema, we
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investigated the effects of PTX treatment on hyperoxia-induced lung injury. We demonstrate that PTX treatment in an experimental model of neonatal hyperoxic lung injury reduces extravascular fibrin deposition and prolongs survival.

MATERIALS AND METHODS

Animals. Timed-pregnant Wistar rats were kept in a 12:12-h dark-light cycle and fed a standard chow diet (Special Diet Services, Witham, Essex, UK) ad libitum. After a gestation of 21.5 days (spontaneous birth occurs 22 days after conception), pregnant rats were killed by decapitation and pups were delivered by hysterectomy through a median abdominal incision. Immediately after birth, pups were dried and stimulated. Pups from two to three litters, with a maximum of 12 pups, were pooled and exposed to 100% oxygen in a transparent 50 x 50 x 70 cm Plexiglas chamber for 10 days or until death occurred (survival experiments). Pups were fed by lactating foster rats. To avoid oxygen toxicity of the foster rats, two groups of five pups exposed to room air were included and used to generate data on room air-exposed controls. Foster rats were rotated daily between the three cages. The oxygen concentration, weight, evidence of disease, and mortality were checked twice a day. Hyperoxia exposed pups were injected subcutaneously, starting on day 2, via a 0.5 ml syringe (U-100 Micro-Fine insulin 29G syringe, Becton Dickinson, NY) at the lower back. Pups received either 150 µl of 75 mg/kg PTX (Sigma, St. Louis, MO) in 0.9% saline or 150 µl of 0.9% saline (age-matched control) twice a day. The research protocol was approved by the Institutional Animal Care and Use Committee of the Leiden University Medical Center.

Tissue preparation. Pups were anesthetized with an intraperitoneal injection of ketamine (50 mg/kg body wt; Ketanest-S, Parke-Davis/Pfizer, New York, NY) and xylazine (50 mg/kg bodyweight; Rompun, Bayer, Leverkusen, Germany). To avoid postmortem fibrin deposition in the lungs, heparin (100 units; Leo Pharma, Breda, the Netherlands) was injected intraperitoneally. After 5 min, pups were exsanguinated by transection of the abdominal blood vessels. The thoracic cavity was opened, and the lungs were removed, snap-frozen in liquid nitrogen, and stored at –80°C until use for real time RT-PCR or the fibrin deposition assay. For histology studies, the trachea was cannulated (Bioflow 0.6 mm intravenous catheter, Vygon, Veenendaal, the Netherlands), and the lungs were fixed in situ via the trachea cannula with buffered formaldehyde (3.8% paraformaldehyde in PBS, pH 7.4) at 25 cmH₂O pressure for 3 min. Lungs were removed, fixed additionally in formaldehyde for 24 h at 4°C, and embedded in paraffin after dehydration in a graded alcohol series and xylene.

Brochoalveolar lavages. Pups were anesthetized with an intraperitoneal injection of ketamine and xylazine and injected intraperitoneally with heparin. A cannula (Bioflow 0.6 mm intravenous catheter) was positioned in
the trachea, and the pups were exsanguinated by transection of the abdominal blood vessels. Lungs were slowly lavaged four times with 500 µl NaCl, 0.9%, 1 mM EDTA (pH 8.0). Samples were pooled and centrifuged for 10 min at 5,000 rpm. Supernatants were stored at -20 ºC until further use.

**Lung histology.** Paraffin sections (4 µm) from the left upper lobe were cut and mounted onto SuperFrost plus-coated slides (Menzel-Gläzer). After deparaffinization, sections were stained with hematoxylin and eosin or with a monoclonal anti-human fibrin antibody (59D8, Boston Research Services, Winchester, MA) that specifically recognizes the β-chain of fibrin (9, 40). For immunohistochemistry, sections were incubated with 0.3% H₂O₂ in methanol to block endogenous peroxidase activity. After a graded alcohol series, sections were boiled in 0.01 M sodium citrate (pH 6.0) for 10 min. Sections were incubated overnight with 59D8, stained with EnVision-HRP (Dako, Glostrup, Denmark), using NovaRed (Vector, Burlingame, CA) as chromogenic substrate, and counterstained briefly with hematoxylin.

**Fibrin detection assay.** Fibrin deposition in lungs was detected as described previously (40). Briefly, frozen lungs were homogenized with an Ultra-Turrax T8 tissue homogenizer (IKA-Werke, Staufen, Germany) for 1 min at full speed in a cold 10 mM sodium phosphate buffer (pH 7.5), containing 5 mM EDTA, 100 mM ε-aminocaproic acid, 10 U/ml aprotinin, 10 U/ml heparin, and 2 mM phenylmethanesulfonyl fluoride. The homogenate was incubated for 16 h on a top over top rotor at 4°C. After centrifugation (10,000 rpm, 4°C, 10 min), the pellet was resuspended in extraction buffer [10 mM sodium phosphate buffer (pH 7.5), 5 mM EDTA, and 100 mM ε-aminocaproic acid] and recentrifuged. Pellets were suspended in 3 M urea, extracted for 2 h at 37°C, and centrifuged at 14,000 rpm for 15 min. After the supernatant was aspirated and discarded, the pellet was dissolved at 65°C in reducing sample buffer (10 mM Tris pH 7.5, 2% SDS, 5% glycerol, 5% β-mercaptoethanol, and 0.4 mg/ml bromophenol blue) for 90 min with vortexing every 15 min. Hereafter, samples were subjected to SDS-PAGE (7.5%; 5% stacking) and blotted onto PVDF membrane (Immobilon-P, Millipore, Bredford, MA). The 56-kDa fibrin β-chains were detected with a monoclonal anti-human fibrin antibody (59D8, Boston Research Services, Winchester, MA) that specifically recognizes β-fibrin (9, 40), using enhanced chemiluminescence (Amersham, Arlington Heights, IL) and Kodak X-OMAT Blue XB-1 films (Kodak, Rochester, NY). Exposures were quantified with a Bio-Rad GS-800 calibrated densitometer using the Quantity One, version 4.4.1 software package (Bio-Rad, Veenendaal, the Netherlands). Fibrin deposition was quantified in lungs of at least four rats per experimental group. As a reference, fibrin standards were generated from rat fibrinogen (Sigma, St. Louis, MO). After rat fibrinogen was solubilized in two-thirds strength PBS (pH 7.4), human α-thrombin (Sigma, St. Louis, MO) was added, vortexed, and incubated at 37°C for 10 min. After addition of 2x SDS sample buffer, the fibrin sample was vortexed and incubated at 65°C for 90 min; aliquots were frozen at -80°C until use.

**Real-time RT-PCR.** Total RNA was isolated from lung tissue homogenates using guanidium-phenol extraction (RNAzol, Campro Scientific,
Veenendaal, the Netherlands). Briefly, after tissue homogenization in RNAzol B, RNA was isolated using phenol-chloroform extraction and isopropanol precipitation. The RNA sample was dissolved in RNase-free water and quantified spectrophotometrically. The integrity of the RNA was studied by gel electrophoresis on a 1% agarose gel, containing ethidium bromide. Samples showing degradation of ribosomal RNA by visual inspection under ultraviolet light were discarded. First-strand cDNA synthesis was performed with the SuperScript Choice System (Life Technologies, Breda, the Netherlands) by mixing 2 µg total RNA with 0.5 µg of oligo(dT)12-18 primer in a total volume of 10 µl. After the mixture was heated at 70°C for 10 min, a solution containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 10 mM DTT, 0.5 mM dNTPs, 0.5 µl RNase inhibitor, and 200 U Superscript Reverse Transcriptase was added, resulting in a total volume of 20 µl. This mixture was incubated at 42°C for 1 h; total volume was adjusted to 100 µl with RNase-free water and stored at –80°C until further use. For real-time quantitative PCR, 1 µl of first-strand cDNA, diluted 1:10 in RNase-free water, was used in a total volume of 25 µl, containing 12.5 µl 2x SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) and 200 ng of each primer. Primers, designed with the Primer Express software package (Applied Biosystems), are listed in Table 1. PCR reactions, consisting of 95°C for 10 min (1 cycle), 94°C for 15 s, and 60°C for 1 min (40 cycles), were performed on an ABI Prism 7700 sequence detection system (Applied Biosystems). Data were analyzed with the ABI Prism 7700 sequence detection system version 1.9 software and quantified with the comparative threshold cycle method with β-actin as a housekeeping gene reference (26).

Protein assay. Total protein concentration was measured in bronchoalveolar lavage fluid (BALF) using the Dc protein assay (Bio-Rad), according to the manufacturer’s instructions. The detection limit was 31 µg/ml.

Statistical analysis. Values are expressed as mean ± SE. Differences between groups were analyzed with the Student’s t-test. For comparison of survival curves, Kaplan-Meier analysis followed by a log rank test was performed. P values of <0.05 were considered statistically significant.
**Table 1.** Sequences of oligonucleotides used as forward and reverse primers for real-time RT-PCR. [MCP, monocyte chemoattractant protein; MMP, matrix metalloproteinase; PAI, plasminogen activator inhibitor; TF, tissue factor.]

**RESULTS**

*Fibrin deposition.* Because fibrin deposition correlates well with the severity of tissue damage in hyperoxia-induced lung injury, we quantified fibrin deposition by Western Blot analysis in lungs after exposure to different oxygen concentrations to determine the optimal experimental conditions for intervention. Fibrin deposition in lung homogenates of preterm rat pups exposed to 60, 80, and 100% oxygen is shown in Figure 1A. Significant fibrin deposition was absent in lungs of pups exposed to 60 and 80% oxygen for 14 days (Figure 1B). However, fibrin deposition increased from 18 ± 8 in room air-exposed pups to 296 ± 42 ng fibrin/mg of tissue in lungs of pups exposed to 100% oxygen for 10 days (Figure 1B; *p* < 0.001), demonstrating that this is the optimal experimental condition. Therefore, intervention studies were performed in preterm rat pups exposed to 100% oxygen. Treatment of oxygen-exposed pups with PTX for 10 days resulted in a reduction of fibrin deposition from 296 ± 42 in oxygen-exposed controls to 100 ± 23 ng fibrin/mg of tissue in lungs of PTX-treated pups exposed to 100% oxygen (Figure 1B; *p* < 0.001). The localization of fibrin deposits in the lungs was studied immunohistochemically on formaldehyde-fixed paraffin sections. Fibrin deposits were mainly observed in the extravascular compartment in the alveolar lumen and were associated with the alveolar wall (Figure 1D). In control lungs, fibrin deposits were absent (Figure 1C).
mRNA expression in lung homogenates. Because PTX has anti-inflammatory, anticoagulant and antifibrinolytic properties, we studied mRNA expression of key enzymes involved in these processes in lung homogenates after lung injury by hyperoxia. TNF-α mRNA expression did not change after oxygen exposure \((p = 0.934)\) or PTX treatment \((p = 0.35; \text{Figure 2A})\). IL-6 \((p < 0.001)\), matrix metalloproteinase-12 (MMP-12; \(p = 0.019\)), and monocyte chemoattractant protein-1 (MCP-1; \(p < 0.001\)) expressions after 10 days of hyperoxia were increased compared with room air-exposed controls (Figure 2A). IL-6 expression \((p = 0.55)\) and MMP-12 expression \((p\)
= 0.29) did not change by PTX treatment during oxygen exposure (Figure 2A). However, MCP-1 mRNA expression decreased 1.6-fold in pups exposed to hyperoxia and 10 days of PTX treatment (Figure 2A; \( p = 0.036 \)). The expression of the physiological initiator of coagulation tissue factor (TF; \( P = 0.008 \)) and the antifibrinolytic factor plasminogen activator inhibitor-1 (PAI-1; \( p < 0.001 \)) increased during exposure to hyperoxia compared to room air-exposed controls (Figure 2B). However, PTX treatment did not change TF (\( p = 0.20 \)) and PAI-1 (\( p = 0.93 \)) expression after exposure to 100% oxygen for 10 days (Figure 2B).

**Figure 2.** Relative mRNA expression, determined with real-time RT-PCR, of genes related to inflammation [TNF-\( \alpha \), IL-6, matrix metalloproteinase-12 (MMP-12), and monocyte chemoattractant protein-1 (MCP-1); A] and coagulation and fibrinolysis [tissue factor (TF) and plasminogen activator inhibitor-1 (PAI-1); B] in 10-day-old room air-exposed pups (white bars), oxygen-exposed pups treated with 0.9% saline (black bars), and oxygen-exposed pups treated with PTX (gray bars). Data are expressed as means ± SE of 6 (room air) or 20 (both oxygen-exposed groups) rats. *\( p < 0.05 \), **\( p < 0.01 \), ***\( p < 0.001 \), oxygen vs. room air-exposed controls. ∆\( p < 0.05 \), PTX vs. oxygen-exposed controls.

**BALF.** Total protein concentration was measured in BALF as a marker for capillary leakage. Protein concentration on postnatal day 10 was 103 ± 7 µg/ml in pups exposed to room air, 328 ± 37 µg/ml in pups exposed to 100% oxygen treated with saline (Figure 3; \( p < 0.001 \)), and 218 ± 18 µg/ml in pups exposed to 100% oxygen treated with PTX (Figure 3; \( p = 0.029 \)). PTX treatment decreased the white blood cell count in BALF from 55 ± 16 x 10^5 cells/ml in oxygen-exposed pups treated with saline to 16 ± 5 x 10^5 cells/ml in oxygen-exposed pups treated with PTX (\( p = 0.05 \)).
Figure 3. Total protein concentration in bronchoalveolar lavage fluid (BALF) of 10-day-old preterm room air-exposed pups injected with 0.9% saline (white bar), oxygen-exposed pups injected with 0.9% saline (black bar), and oxygen-exposed pups injected with PTX (gray bar). Data are expressed as means ± SE of 5 rats per group. ***p < 0.001, oxygen vs. room air-exposed controls. Δp < 0.05, PTX vs. oxygen-exposed controls.

Survival. Survival of PTX-treated hyperoxia-exposed pups was prolonged compared with oxygen-exposed controls (Figure 4; p = 0.0018). After 12 days of oxygen exposure, 73% of the controls had died vs. only 20% of the PTX pups. The mean survival of preterm oxygen-exposed rat pups treated with PTX was prolonged by 3 days. Subcutaneous injection of room air-exposed pups (n = 5) with 0.9% saline did not lead to illness or mortality the first 4 wk after birth (data not shown).

Figure 4. Kaplan-Meier survival curve of preterm rats treated with either 0.9% saline (Δ, n = 26) or 75 mg/kg PTX twice a day (●, n = 25) exposed to 100% oxygen. Data are expressed as percentage ± SE of pups surviving at the observed time point. p < 0.01, PTX vs. saline controls.
DISCUSSION

The reported experiments show that PTX treatment reduces fibrin deposition threefold and prolongs survival by 3 days in preterm rat pups with hyperoxia-induced lung injury. Fibrin deposition is an important contributor to the pathogenesis of lung injury by oxidative stress. The findings of this study suggest that inhibition of fibrin deposition may have therapeutic potential in the treatment of BPD. This hypothesis is supported by studies in PAI-1 knock-out mice, which have less hyperoxia-induced fibrin deposition, resulting in a less severe phenotype and increased resistance toward hyperoxia-induced mortality (2). In addition, blocking of the coagulation cascade attenuates acute lung injury induced by gram-negative sepsis in baboons (4). Fibrin may have proinflammatory and profibrotic properties, by facilitating cell migration and activating inflammatory cells and fibroblasts (31), and it hampers pulmonary gas exchange via inactivation of lung surfactant (7, 30).

Because the mechanism by which PTX reduces fibrin deposition and prolongs survival can be multifactorial, we studied the mRNA expression of key enzymes involved in inflammation, coagulation, and fibrinolysis. PTX reduced MCP-1 mRNA expression but not the expression of TNF-α, IL-6, and MMP-12, indicating that only the influx of monocytes and macrophages to the lung is inhibited during the inflammatory response. PTX did not reduce mRNA expression of TF and PAI-1, indicating that transcriptional activation of the coagulation cascade and inhibition of the fibrinolytic cascade are similar under these experimental conditions.

The effects of PTX treatment in hyperoxia-induced lung injury on the transcriptional regulation of inflammation and coagulation are minor. Because fibrin deposits are located in the extravascular compartment in alveolar septa and lumen, extravasation of plasma proteins, including fibrinogen, into the alveolar lumen may be important. This is supported by a decrease in protein concentration in BALF after PTX treatment, indicating that extravasation of plasma proteins is inhibited by PTX, probably resulting in a lower fibrinogen content in the alveolar fluid and, as a result, less fibrin deposition.

Conflicting results have been found on survival after PTX treatment in lung injury models. In contrast to our findings, PTX treatment did not prolong survival in adult rats after exposure to >95% oxygen (25). This discrepancy may be explained by the difference in oxygen tolerance between neonates and adults in combination with a lower PTX dosage used in the adult rat experiments. Beneficial effects of PTX treatment on survival were reported after endotoxemia, even at low dosages, in mice (42).

We only found an effect of PTX on MCP-1 mRNA expression but not on TNF-α and IL-6 expression. Many other in vitro and in vivo studies demonstrate that PTX inhibits the expression of proinflammatory cytokines (6, 19, 23, 24, 29, 32, 34, 35, 42). These studies emphasize the attenuating effect of PTX on TNF-α and IL-6, the key proinflammatory targets of PTX. Moreover, three studies implicate that inhibition of the release of TNF-α is
the most important outcome predictor in endotoxemia (24, 34, 42). A possible explanation for this discrepancy might be that TNF-α does not play the same pivotal role in the inflammatory response in neonatal hyperoxia-induced lung injury as it does in endotoxemia. The finding that TNF-α was not significantly different between room air-exposed and oxygen-exposed pups strengthens this hypothesis. In contrast, the importance of MCP-1 has been demonstrated in a hyperoxia-induced BPD model. Newborn rats exposed to hyperoxia were injected with anti-MCP-1, resulting in the prevention of neutrophil influx and reduced protein oxidation (36). We assume that the inhibitory effect of PTX on the expression of MCP-1 results in a reduction of the influx of macrophages and/or neutrophils to the lung, resulting in less tissue damage and contributing to an improvement in BPD.

The clinical importance of PTX treatment was recently demonstrated by preliminary data on preterm neonates, prone to develop BPD, in whom pretreatment with 40-80 mg·kg\(^{-1}\)·day\(^{-1}\) of nebulized PTX in four dosages reduced treatment requirements after the first month of life (21). Limitations of the potential clinical importance of our observations are the high oxygen concentration (100%) used for a prolonged period (10 days) to induce lung injury and the relatively high PTX concentration compared with dosages used in the clinic: 150 mg·kg\(^{-1}\)·day\(^{-1}\) subcutaneously in our study vs. 40-80 mg·kg\(^{-1}\)·day\(^{-1}\) by nebulization and 30 mg·kg\(^{-1}\)·day\(^{-1}\) intravenously in preterm human infants (20, 21). Furthermore, few human preterm infants will be exposed to 100% oxygen for 10 days, but less extreme oxygen exposures did not produce the primary indicator of injury (fibrin deposition) in the animal model used in this study.

In summary, this study shows that PTX significantly prolongs survival and attenuates alveolar fibrin deposition of preterm rat pups with experimental neonatal hyperoxic lung injury. The effect of PTX is probably the result of decreased protein leakage from the capillaries to the alveolar lumen. PTX may have the potential to prevent and/or reduce the severity of BPD in preterm infants who need ventilatory support in the neonatal intensive care unit.

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