Pentoxifylline does not protect against hyperoxic lung injury in rats

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ABSTRACT: Hyperoxia has been used extensively as a model of acute lung injury. The drug pentoxifylline has been shown to have a protective effect in other models of lung injury. We sought to determine whether pentoxifylline protects against hyperoxic lung injury in rats by decreasing the accumulation of neutrophils within the lung.

A total of 84 rats were studied. Twenty four rats were randomized into four groups. Two groups of rats were pretreated for 48 h with either pentoxifylline (20 mg·kg⁻¹) or saline, and then exposed to >95% O₂ for 60 h while treatments continued. Two groups of control rats received the same treatment regimens as the O₂-exposed animals, but breathed room air. Neutrophil accumulation in the lung was quantified both by histology and myeloperoxidase activity.

Lung neutrophil accumulation increased in the oxygen-exposed group receiving pentoxifylline as compared to oxygen- or air-exposed rats receiving saline injections. Total glutathione was higher in lung homogenates from the hyperoxic, pentoxifylline-treated group than in homogenates from the other three groups. To study survival, 60 rats were exposed to >95% O₂ for 120 h, 30 rats were pretreated with pentoxifylline, and 30 received saline. Survival after 120 h of exposure to hyperoxia was not altered by pentoxifylline treatment (pentoxifylline treated: 6 out of 30 survived; saline treated: 2 out of 30 survived).

We conclude that pentoxifylline does not reduce mortality or lung injury in rats exposed to hyperoxia and is associated with an increase in lung neutrophil accumulation.


The toxic effects of hyperoxia upon the lungs are well-known [1–3]. In adult rats, lung injury becomes evident after 50 h, and death usually occurs within 72 h of exposure to 100% oxygen [1]. Neutrophils accumulate in the lungs of rats soon after exposure to 100% oxygen, and may play a role in this form of acute lung injury [4, 5].

Pentoxifylline, a methylxanthine, has been shown to be protective in models of acute lung injury due to sepsis or endotoxin, in part by preventing the sequestration of neutrophils within the pulmonary circulation [6, 7]. It has been postulated that pentoxifylline inhibits the effect of tumour necrosis factor (TNF) on neutrophils [8–10], and/or inhibits monocyte synthesis of TNF in response to endotoxin [11]. Pentoxifylline exerts its effects on neutrophils at the cell surface through a mechanism that has yet to be characterized. SULLIVAN and co-workers [8] have shown that pentoxifylline restores TNF-inhibited neutrophil migration. Adenosine has been shown to exert a similar effect. Pentoxifylline, however, does not act through either of the known adenosine receptors, as specific antagonists of the A₁ and A₂ receptors do not block the effect of either adenosine or pentoxifylline [12].

In the present investigation, we tested whether intra-peritoneal pentoxifylline affects lung injury and mortality by decreasing the accumulation of neutrophils in the lungs of hyperoxic rats. Lactate dehydrogenase (LDH) activity and albumin concentration in bronchoalveolar lavage fluid (BALF) were measured as indices of lung injury. Neutrophil accumulation in the lung was measured by histological examination and by myeloperoxidase assay. Total glutathione was also measured in lung tissue as an index of oxidative stress. Our results suggest that, in the rat hyperoxic model of lung injury, pentoxifylline does not reduce the accumulation of neutrophils within the lungs, nor does it protect against lung injury or decrease mortality.

Materials and methods

Study animals

Eighty four male 275–325 g Sprague-Dawley rats were used. All animal experiments conformed to the Helsinki convention for the use and care of animals.
Study design

To determine whether pentoxifylline decreased hyperoxia-induced mortality, we pretreated rats for 48 h with intraperitoneal injections of either pentoxifylline or an equal volume of saline. The animals were then placed in a >95% O₂ atmosphere, whilst treatment with either pentoxifylline or saline was continued. Mortality was determined at the end of 120 h of exposure.

To determine whether pentoxifylline reduced lung injury caused by sublethal oxygen exposure, we pretreated rats with either pentoxifylline or saline, as described above. The rats were then exposed to either air or >95% O₂ for 60 h, before being sacrificed. This time interval was chosen as survival rate was almost 100% at 60 h, in this model. Measurements of neutrophil accumulation within the lung were made using an assay of the enzyme myeloperoxidase, and by direct counting of histological sections. The lung tissue was also assayed for total glutathione as a index of oxidant injury. BALF was also obtained from each rat and assayed for cell count, albumin and lactate dehydrogenase (LDH).

Mortality studies

Sixty 275–325 g male Sprague-Dawley rats were randomized into two groups. Thirty were pretreated for 48 h with pentoxifylline (generously supplied by Hoechst-Roussel) (20 mg·kg⁻¹ b.i.d. by intraperitoneal (i.p.) injection). The other thirty were injected with an equivalent volume of normal saline. Both groups were placed in a >95% O₂ environmental chamber (Kirchner Co.) and given food and water ad libitum, whilst injections of pentoxifylline or saline were continued. The number of deceased rats were counted every 12 h for up to 120 h.

Injury studies

Twenty four male Sprague-Dawley rats were randomized into four groups. They received i.p. injections of 20 mg·kg⁻¹ of pentoxifylline or an equivalent volume of saline every 12 h for 48 h. Eight rats receiving pentoxifylline injections and 8 rats receiving saline injections were then placed in a >95% O₂ environmental chamber for 60 h. The remaining 4 pentoxifylline-treated, and 4 saline-treated rats were maintained in room air. In all groups, treatment with pentoxifylline or saline was continued. The rats in all four groups were allowed food and water ad libitum. At the end of 60 h, the rats were given a lethal injection of pentobarbital, heparinized, and exsanguinated by cannulation of the inferior vena cava. A sample of blood was saved for neutrophil isolation as described below. The heart and lungs of each rat were removed en bloc. The lungs were perfused with a phosphate buffered saline solution (pH 7.4) containing 3 g·dl⁻¹ albumin, at a pressure of 20 cmH₂O. Perfusion was stopped when the fluid emerging from the cannula in the inferior vena cava was completely clear (approximately 20 ml of perfusate). After ligation of the left main-stem bronchus, the right lung was slowly lavaged with 10 ml of a 4°C saline solution, taking care not to exceed 15 cmH₂O of airway pressure. The lavage fluid was spun for cell count, and measurement of LDH and albumin. The lungs were dissected away from the heart, great vessels, and large airways, and portions of the left lung, which had not been subjected to BAL, were analysed for total glutathione (GSH), and myeloperoxidase activity, as described below.

Myeloperoxidase assay

To assess lung neutrophil number, myeloperoxidase (MPO) was measured using a modification of a method described previously [13]. In brief, 500 mg samples of lung from each animal was subjected to three cycles of homogenization in 2.5 ml of 50 mM potassium phosphate buffer (pH 6.0) with 0.5% hexadecyltrimethylammonium bromide (HTAB), freezing in a methanol bath, and centrifugation at 2,000×g (20 min). The three supernatants were combined, passed through a 0.22 µm millipore filter and assayed for myeloperoxidase activity. To normalize lung myeloperoxidase to neutrophil number, neutrophils from each rat were isolated from peripheral blood samples using Ficoll gradient sedimentation, followed by osmotic erythrocyte lysis. The neutrophil concentration was determined using a haemocytometer. The neutrophils were then suspended in 2.0 ml of 0.5% HTAB/potassium phosphate buffer (50 mM, pH 6.0), subjected to three cycles of freezing in a methanol/dry ice bath, centrifuged at 2,000×g, and assayed for myeloperoxidase activity. A standard curve of myeloperoxidase activity versus neutrophil number was then determined for the neutrophils from each animal. A representative standard curve is shown in figure 1. MPO was assayed spectrophotometrically by adding a 50 µl sample to 1.40 ml of 50 mM phosphate buffer (pH 6.0) containing 0.167 mg·ml⁻¹ o-dianisidine dihydrochloride. Hydrogen peroxide was added to bring the final concentration of H₂O₂ to 0.0005%. The rate of change in absorbance was measured at 460 nm in a Beckman Model 35 spectrophotometer.

![Fig. 1.](image-url) — Representative standard curve of myeloperoxidase activity (arbitrary units (a.u.)) versus neutrophil number.
Neutrophil counts

In order to independently verify the results of the myeloperoxidase assay, the right upper lobes from the rats in all four groups were fixed in formaldehyde and representative sections were prepared. The number of neutrophils contained in 10 alveoli was counted in 10 randomly chosen fields per rat by a pathologist (V.S.) blinded to treatment conditions.

Glutathione assay

Total glutathione was measured using a previously described protocol [14], where 0.25 g of lung was homogenized in 5 ml of 10 mM 5,5'-dithiobis 2-nitrobenzoic acid (DTNB), 5 mM ethylene-diamine tetra-acetic acid (EDTA), 100 mM potassium phosphate buffer, pH 7.4 (Buffer A). Total glutathione was measured by adding 500 ml of homogenate to 500 ml of 10 mM DTNB. The proteins were removed from the sample by precipitation with 500 ml of 4% sulphosalicylic acid (SSA), followed by centrifugation at 2,000 × g for 5 min. Fifty millilitres of the sample was then mixed in a cuvette with 50 ml of DTNB solution 0.5 U glutathione (oxidized form) (GSSG) reductase and 115 nmol nicotinamide adenine dinucleotide phosphate (reduced form) (NADPH) in a 100 mM potassium phosphate buffer (pH 7.4) to a total volume of 900 ml. The change in absorption at 412 nm with respect to time was then measured and referenced to a standard concentration of GSH.

Bronchoalveolar lavage measurements

To assess the effect of pentoxifylline on lung injury, BALF cell count (number·ml⁻¹) was determined by counting 10 ml of BALF using a haemocytometer. All counts were made in quadruplicate. To assess capillary permeability, albumin was determined spectrophotometrically using Sigma (St. Louis, MO, USA) kit No. 625-2, and LDH was determined using Sigma assay No. dg 1340-K.

Statistical analysis

Results are expressed as mean±standard deviation. All group comparisons were made using one way analysis of variance (ANOVA), with a comparison of means by the Duncan method. The mortality comparisons were made by Chi-squared test.

Results

Mortality study

As shown in figure 2, pentoxifylline did not reduce mortality in rats exposed to hyperoxia. Six out of 30 pentoxifylline-treated rats survived 120 h of >95% O₂, whilst 2 out of 30 saline-treated rats survived the same challenge (p=0.10).

Measurement of lung injury

As shown in table 1, cellularity of the BALF of oxygen-exposed animals was significantly increased over the levels seen in normoxic animals (p<0.01). Pentoxifylline treatment did not independently change BALF cell count in either hyperoxic or normoxic animals. BALF albumin levels were elevated in the hyperoxia-exposed rats, whether receiving pentoxifylline or saline, over both normoxic groups (p<0.05), but were no different from one another. Levels of LDH in hyperoxic, pentoxifylline- or saline-treated rats, were greater than in either of the normoxic control groups (p<0.05), but were not different from each other.

As shown in figure 3, total GSH levels were higher in both oxygen-exposed groups when compared to the normoxic controls (p<0.05). Pentoxifylline treatment increased the GSH levels in hyperoxia-exposed rats over the levels seen in saline-treated rats exposed to hyperoxia (p<0.05). Total GSH levels did not differ between normoxic pentoxifylline-treated and normoxic saline-treated rats.

Table 1. – Effect of hyperoxia and pentoxifylline on cell count, albumin and LDH measured in bronchoalveolar lavage fluid

<table>
<thead>
<tr>
<th>Group</th>
<th>Cell count cells·ml⁻¹</th>
<th>Albumin mg·dl⁻¹</th>
<th>LDH IU·dl⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pentoxifylline/O₂</td>
<td>34.0±8.6*</td>
<td>48.4±10.7*</td>
<td>29.9±6.1*</td>
</tr>
<tr>
<td>Saline/O₂</td>
<td>45.9±7.2*</td>
<td>29.5±6.1*</td>
<td>23.6±3.7*</td>
</tr>
<tr>
<td>Pentoxifylline/air</td>
<td>3.3±2.2</td>
<td>6.1±2.6</td>
<td>5.9±2.9</td>
</tr>
<tr>
<td>Saline/air</td>
<td>2.7±1.5</td>
<td>13.5±9.9</td>
<td>9.2±2.9</td>
</tr>
</tbody>
</table>

Data are presented as mean±standard deviation. *, **: difference (p<0.05, <0.01) as compared to rats breathing room air; LDH: lactate dehydrogenase.
Neutrophil accumulation

As assessed by myeloperoxidase activity (fig. 4a), the number of neutrophils was higher in hyperoxic animals receiving treatment with pentoxifylline than in those receiving saline treatment or saline-treated normoxic animals (p<0.05). The number of neutrophils in the pentoxifylline-treated normoxic group tended to be higher but was not different from the normoxic, saline-treated rats (p=0.08).

The neutrophil numbers per alveoli, assessed by direct counting (fig. 4b), support the myeloperoxidase measurements. Hyperoxic rats treated with pentoxifylline had more lung neutrophils than saline-treated animals exposed to hyperoxia (p<0.05). Both saline-O2 and pentoxifylline-O2 rats had significantly greater numbers of neutrophils per alveolus than normoxic animals receiving either pentoxifylline or saline treatments (p<0.01). Also, among room air-exposed rats, those treated with pentoxifylline had increased lung neutrophil accumulation (p<0.05).

Discussion

Hyperoxia has been used extensively as an animal model of acute lung injury. As the drug pentoxifylline has been found to be protective in other models of acute lung injury, we investigated whether it would ameliorate hyperoxia-induced lung injury and mortality in rats. We sought to determine whether pretreatment with this drug prevented the accumulation of neutrophils within the rat lung in our model, an effect well-documented in other lung injury models [6, 7].

We observed that pentoxifylline did not protect against hyperoxic toxicity in rats. Mortality was similar in the pentoxifylline- and saline-treated rats exposed to hyperoxia. The degree of lung injury as assessed by BALF cell counts, BALF LDH activity, and BALF albumin concentration, was not reduced by pentoxifylline treatment in hyperoxia-exposed rats. In contrast to the effect seen with pentoxifylline, in endotoxin-induced lung injury, we found an increase in the number of lung neutrophils in hyperoxic animals treated with pentoxifylline. In endotoxin-induced injury, pentoxifylline decreases the number of neutrophils within the lung, with a concomitant reduction in lung injury [6, 7]. The contrasting responses to pentoxifylline in these two models of lung injury may be due to a difference in the role played by the neutrophil in each. In endotoxin-induced lung injury, damage is thought to be mediated through a number of cytokines, including TNF [15–17]. These agents act as potent activators of circulating neutrophils, inducing increased endothelial adhesion, degranulation and oxidative burst, as well as decreased chemotaxis [18, 19]. Evidence suggests that pentoxifylline protects against endotoxin-induced lung injury by counteracting these effects of TNF upon neutrophils [9–12, 20].

In hyperoxia-induced lung injury, a variety of cells, such as alveolar macrophages, produce chemotaxins. This results in the attraction and sequestration of neutrophils within the lung; neutrophils then produce lung injury by degranulation and free radical and proteolytic enzyme release [21, 22]. The lack of a protective effect of pentoxifylline in hyperoxic lung injury possibly suggests that TNF is not a major contributor in hyperoxia. Although neutrophils have been shown to contribute to
lung injury in hyperoxia [4, 5], there is also data to suggest that hyperoxic lung injury may occur in the absence of neutrophils, as shown in a previous study, where neutrophil depletion by cyclophosphamide did not affect lung edema in rats exposed to hyperoxia [23]. The production of free radicals has been shown to contribute significantly to hyperoxia-induced lung injury, and a number of studies have shown an improvement in survival with the use of free radical scavengers. WHITE et al. [24] demonstrated improved survival in rats pretreated with superoxide dismutase (SOD) and catalase (CAT) attached to polyethylene glycol. JACOBSON et al. [25] demonstrated a similar effect of conjugated SOD and CAT in a hyperoxic rabbit model. They also demonstrated a protective effect with vitamin E and hydroxianisol in this model.

Pentoxifylline has also been shown to counteract TNF inhibition of chemotaxis [12]. SULLIVAN and co-workers [17] reported enhancement of chemotaxis with pentoxifylline alone in the absence of TNF inhibition. The increase in accumulated neutrophils seen in the pentoxifylline-treated animals in our study may be explained by a greater migration of neutrophils into an area already damaged by free radical production. The accumulation of glutathione has been shown to be a marker of increased oxidative stress both in hyperoxic and endotoxic lung injury [13, 26–28]. In our study, pentoxifylline was associated with increased glutathione concentrations in hyperoxia-exposed rats. This effect may be due either to increased oxidative stress from the elevated number of neutrophils or to a direct, and yet unknown, effect of pentoxifylline.

There were a number of limitations of the study which may, in part, explain the negative results. If an insufficient dose of pentoxifylline were given, a higher dose of drug may result in a protective effect. However, when the doses used in our study were compared to other models showing a protective effect of pentoxifylline, this did not appear to be the case. A wide range of doses have been used in various models of lung injury. As the half life of intravenous pentoxifylline has been shown to be less than 2 h in humans [29], continuous i.v. infusions have been utilized by some investigators. The large numbers of animals required for this study and the difficulties inherent in continuous catheterization in rats made this choice impractical for this study. A number of studies have shown a protective effect of pentoxifylline with intermittent dosing. FLETCHER et al. [30] demonstrated that a single 20 mg·kg⁻¹ i.p. dose of pentoxifylline, administered 15 min after an intravenous lipopolysaccharide (LPS) infusion, significantly improved survival. In the same study, higher doses (100 mg·kg⁻¹ i.p) resulted in decreased survival. An earlier study by CHALKIADAKIS et al. [31] demonstrated a protective effect by pentoxifylline in a rat peritonitis model. A dose of 17 mg·kg⁻¹ given intramuscularly once a day resulted in 2 deaths out of 20 rats, compared to 16 deaths out of 20 animals not receiving pentoxifylline pretreatment.

Another limitation of the study is that nonlabelled albumin is a relatively insensitive index of lung injury. However, when considered together with the other indices of lung injury used, including BALF LDH and BALF cell count, the results indicate that pentoxifylline did not attenuate lung injury in this model.

In summary, this study shows that pentoxifylline treatment in rats does not reduce mortality or lung injury induced by hyperoxia. Pentoxifylline treatment does, however, increase neutrophil accumulation, possibly by increasing chemotaxis induced by factors other than TNF. This results in increased oxidative stress as evidenced by elevated total glutathione in the pentoxifylline treated rats.

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References