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ORIGINAL ARTICLE

Azithromycin reduces airway inflammation in a murine model of lung ischaemia reperfusion injury

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Summary

Clinical studies revealed that azithromycin reduces airway neutrophilia during chronic rejection after lung transplantation. Our aim was to investigate the possible effect of azithromycin on ischaemia-reperfusion injury. Azithromycin or water was administered to mice every other day during 2 weeks ($n = 6$/group). On the 14th day, the left lung was clamped to induce ischaemia (90 min). In two additional groups, animals underwent the same protocol, followed by 4 h of reperfusion. Two control groups were included with thoracotomy only. Inflammatory parameters and oxidative stress were measured in broncho-alveolar lavage of the left lung. Leukocytes, lymphocytes, neutrophils, 8-isoprostane and IL-1β levels after ischaemia and reperfusion were significantly reduced in mice treated with azithromycin. There was a trend towards lower IL-6 and KC levels. A significant correlation was seen between 8-isoprostanes and neutrophils (Pearson $r = 0.72$; $P = 0.0086$), IL-6 (Pearson $r = 0.84$; $P = 0.0006$), KC (Pearson $r = 0.88$; $P = 0.0002$) and IL-1β (Pearson $r = 0.62$; $P = 0.0326$). We conclude (i) that azithromycin reduces inflammation and oxidative stress in our IRI model, and (ii) that oxidative stress is correlated with the number of neutrophils and IL-6, KC and IL-1β levels after ischaemia and reperfusion. Azithromycin should be further investigated as a novel drug to prevent lung ischaemia-reperfusion injury.

Introduction

Lung transplantation (LTx) has enjoyed increasing success over the last decades because of advances in surgical technique, improved organ preservation and better immunosuppression. It has evolved from an experimental endeavor to the mainstay of therapy for many patients suffering from end-stage lung diseases. Some critical issues in LTx, however, still persist, like donor organ shortage, ischaemia reperfusion injury (IRI) and chronic rejection. IRI is responsible for 30% of the postoperative mortality in patients within the first month after LTx [1]. IRI occurs in a biphasic pattern [2]. The early phase of reperfusion primarily depends on donor characteristics, losing importance during the ensuing 24 h, with recipient characteristics becoming predominant thereafter. Donor macrophages have been shown to be activated during ischaemia, thereby mediating the early phase of reperfusion injury, whereas neutrophils are primarily involved in the late phase of reperfusion (starting at 4 h after the onset of reperfusion) [3,4].

Bronchiolitis Obliterans Syndrome (BOS) hampers long-term survival after LTx [1]. BOS is usually treated by augmenting or switching immunosuppressive therapy,

resulting in, at the best, a temporary stabilization of lung function [5,6]. Recently, azithromycin (AZI), a macrolide antibiotic, was shown to improve lung function with 15% on average in 42% of the patients [7–9]. This improvement was accompanied by a reduction in BAL neutrophils and IL-8, suggesting that the efficacy of AZI lies within its anti-inflammatory effects [10] that have already been demonstrated in other pathologies like panbronchiolitis, chronic obstructive pulmonary disease and cystic fibrosis [11–13]. Recently, our colleagues demonstrated that AZI can also reduce oxidative stress in an *in vitro* model [14].

In previous studies in a murine IRI model, we observed an increase in broncho-alveolar macrophages and lymphocytes during the ischaemic period [15]. The rise in cells became significant after 60 min of warm ischaemia.

In this study, we investigated the possible anti-inflammatory effects of AZI on IRI, characterized by a neutrophil influx in the alveolar space. The second aim was to explore the underlying mechanisms, looking at inflammatory cytokine, chemokine and growth factor levels and oxidative stress (by measuring 8-isoprostane levels).

Materials and methods

Animal preparation

Female SWISS outbred mice (20–25 g), obtained from Janvier (Savigny/Orges, France) were used. All animals received humane care in compliance with the 'Principles of Laboratory Animal Care' formulated by the National Society for Medical Research and the 'Guide for the Care and Use of Laboratory Animals', prepared by the Institute for Laboratory Animal Resources and published by the National Institutes of Health (NIH Publication No 86-23, revised 1996). The study was approved by the Institutional Review Board on animal research at the Katholieke Universiteit Leuven.

Animals were housed in cages under specific pathogen free conditions and fed *ad libitum* with a standard diet. Animals were anaesthetized with an intraperitoneal injection of 60 mg/kg pentobarbital (Nembutal®; Sanofi, Brussels, Belgium). Mice were placed on a heating pad at 37 °C (Harvard Apparatus, Holliston, MA, USA) to maintain body temperature with rectal temperature monitored during the entire experiment. Endotracheal intubation was performed through a tracheotomy. Animals were mechanically ventilated (120 strokes/min, 320 μl stroke volume) with room air using a MiniVent type 845 ventilator (Hugo Sachs Elektronik, March-Hugstetten, Germany).

Experimental groups

Animals were randomly assigned to six groups (36 animals, six per group). Water (H_2O) or AZI (125 μl, 3.8 mg/kg in water) was administered to mice by gavage every other day for 2 weeks. On the 14th day, a left thoracotomy was performed and the hilum of the left lung, including bronchus, pulmonary artery and pulmonary vein, was occluded using a noncrushing microsurgical clamp for 90 min in two ischaemia groups ([H_2O 90I] and [AZI 90I], respectively). During ischaemia of the left lung, the animal survived on the contralateral lung. In two more groups, the animals underwent the same protocol, followed by 4 h of reperfusion ([H_2O 90I + R] and [AZI 90I + R], respectively).

To investigate the effect of AZI on normal lungs, two control groups were included, where a thoracotomy only was performed ([H_2O control] and [AZI control], respectively).

At the end of the experiment, all animals were killed.

Broncho-alveolar lavage

Broncho-alveolar lavage (BAL) was performed immediately after sacrifice of the animals with the right lung clamped to obtain BAL from the left lung only. Four aliquots of 0.5 ml sterile saline at room temperature were instilled in the trachea. The returned fractions were centrifuged immediately (500 *g*, 10 min, 4 °C). The supernatant of the first lavage was used for protein measurements (cytokines, chemokines and growth factors) and oxidative stress (8-isoprostanes). The supernatant of the three other lavages was discarded. The pellet of the four lavages was pooled and used for total and differential cell count. BAL supernatant samples were immediately frozen and stored at −80 °C until assayed.

Cell counts

Cell pellets were resuspended in 250 μl of saline for total cell count. Cells were stained with a 0.4% trypan blue solution (Sigma-Aldrich, Irvine, UK) and counted in triplicate using a Bürker chamber. After total cell count, an additional 750 μl of saline was added to the dissolved pellet. A cytospin was made using a Shandon cytocentrifuge (Techgen, Zellik, Belgium). The cytospins were stained using Diff-Quik (Dade Behring, Newark, NJ, USA) to perform differential cell counts on at least 300 cells.

Measurement of IL-1β protein levels in BAL

Mouse IL-1β levels were measured in undiluted BAL supernatant of all groups, using a commercially available ELISA kit as specified by the manufacturer (Biosource International, Nivelles, Belgium). The sensitivity of the cytokine ELISA is 7 pg/ml.

Multiplex Cytometric Bead Array

Mouse IFN-γ, TNF-α, IL-2, GM-CSF, KC, MCP-1 and IL-6 were measured in BAL, using a commercially available Multiplex Cytometric Bead Array Flex kits as specified by the manufacturer (BD Biosciences, Erembodegem, Belgium). The sensitivity of the different assays is 5.2 (IFN-γ), 17.1 (TNF-α), 1.5 (IL-2), 9.9 (GM-CSF), 16.2 (KC), 29.0 (MCP-1) and 6.5 pg/ml (IL-6).

Oxidative stress

Oxidative stress was analyzed by measuring 8-isoprostane levels using a commercially available EIA assay with a detection limit of 5 pg/ml (Cayman, Tallinn, Estonia).

Lung biopsies

Once BAL was obtained, the left lung was excised. A piece of the lateral part of each lung was stored in 0.4% buffered formalin solution. Tissue samples were then embedded in paraffin. Sections were prepared and stained with haematoxylin and eosin for histological analysis.

Statistical analysis

Data analysis was performed with Graphpad Prism 4 (San Diego, CA, USA). A D'Agostino and Pearson omnibus normality test was performed. All data were found to be normally distributed. Results are presented as mean (±SD). A one-way ANOVA (three groups) or an unpaired *t*-test were (two groups) used to look for differences between the groups. Correlation analysis was performed on data from [H_2O 90I + R] and [AZI 90I + R] ($n = 12$ animals) using a Pearson correlation test. A *P*-value of <0.05 was considered significant.

Results

At the cellular level, the number of leukocytes in BAL was significantly lower after ischaemia and reperfusion in animals treated with AZI versus H_2O. This difference was not seen in the control and also not in the ischaemia groups (Fig. 1a). The number of leukocytes was significantly higher in both reperfusion groups compared with that in control or ischaemia groups.

There were no significant differences in the number of macrophages between AZI- and H_2O-treated animals in the different experimental settings (Fig. 1b). Macrophage numbers were significantly higher in both reperfusion groups than in control or in ischaemia groups.

Lymphocyte and neutrophil numbers were different between both reperfusion groups, with lower numbers after AZI pretreatment, but not in the ischaemia and control groups (Fig. 1c and d). Lymphocytes were significantly higher in [H_2O 90I + R] than in control and ischaemia groups. The number of neutrophils increased significantly only after reperfusion compared to control and ischaemia groups.

At the protein level, IL-1β was significantly lower after ischaemia and reperfusion in the group pretreated with AZI than in the group treated with H_2O (Fig. 2a). As a

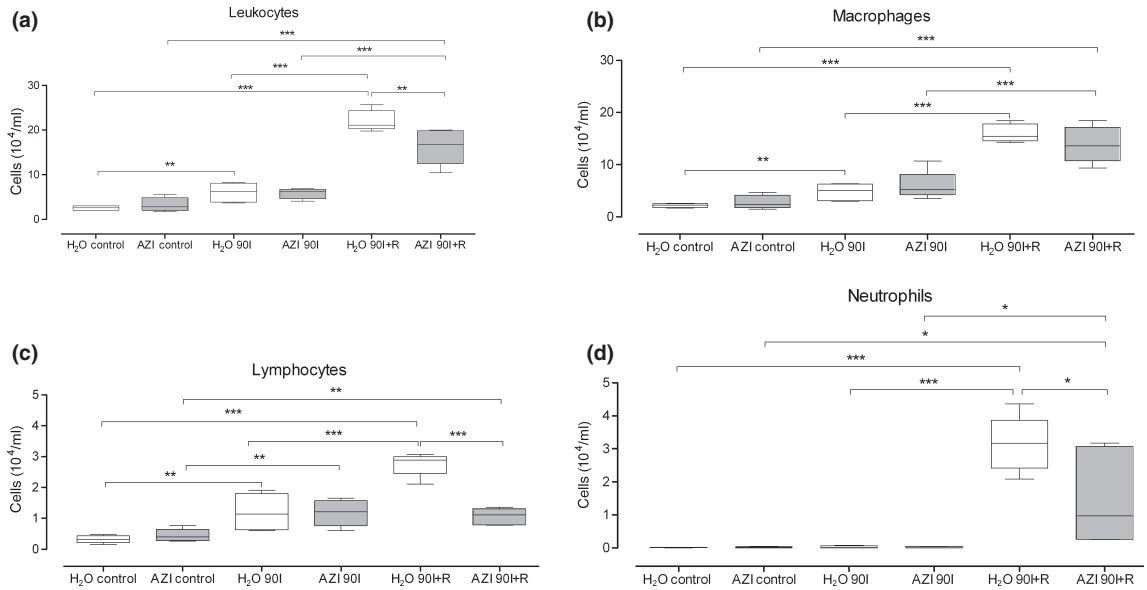

Figure 1 Cell counts in BAL of the left lung in the different study groups. Leucocytes (a), macrophages (b), lymphocytes (c), neutrophils (d). $*P < 0.05$; $**P < 0.01$, $***P < 0.001$. I, ischaemia; R, reperfusion; AZI, azithromycin.

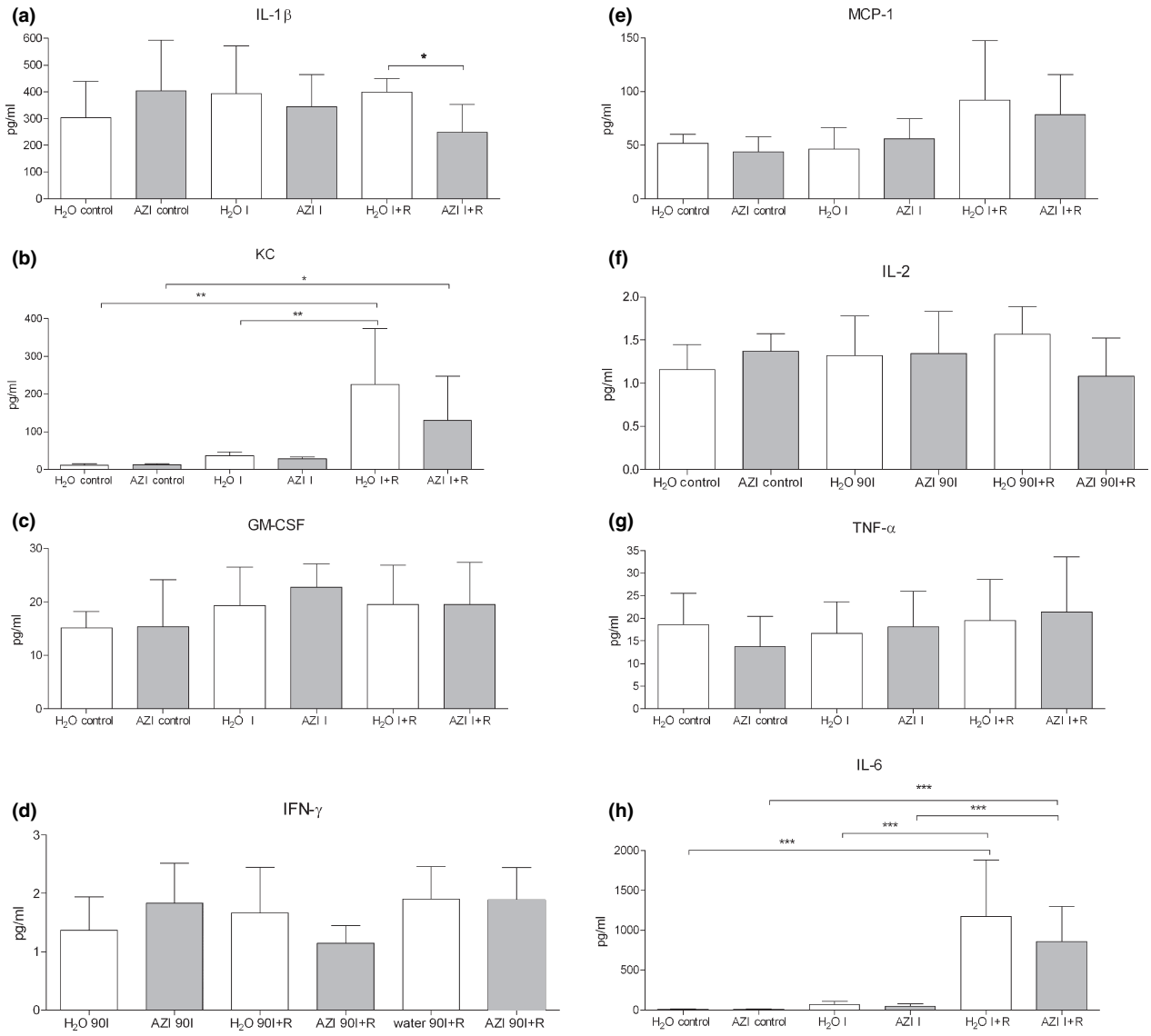

Figure 2 Cytokine, chemokine and growth factor levels in BAL of the left lung in the different study groups: IL-1β (a), KC (b), GM-CSF (c), IFN-γ (d), MCP-1 (e), IL-2 (f), TNF-α (g), IL-6 (h). *$P < 0.05$; **$P < 0.01$; ***$P < 0.005$. I, ischaemia; R, reperfusion; AZI, azithromycin.

result of large standard deviations, there were no significant differences between both reperfusion groups for MCP-1 ($P = 0.63$), KC ($P = 0.25$) and IL-6 ($P = 0.37$). IL-6 and KC levels in BAL increased significantly after reperfusion in both groups.

At the level of oxidative stress, isoprostane levels increased after ischaemia and further increased after reperfusion (Fig. 3). Isoprostane levels were significantly lower in animals treated with AZI than in animals treated with H_2O, after ischaemia as well as after ischaemia and reperfusion.

Correlation analysis between inflammatory cells, cytokines, chemokine, growth factor and oxidative stress in the reperfusion groups is listed in Table 1. 8-Isoprostane levels are significantly correlated with neutrophil numbers, IL-6, KC and IL-1β levels. IL-1β levels also correlated positively with numbers of macrophages and lymphocytes.

Haematoxylin and eosin stained sections show normal lungs in control animals (Fig. 4a and b). Sections of lungs with 90 min of ischaemia show slightly more leucocytes (Fig. 4c and d). In the reperfusion groups, the

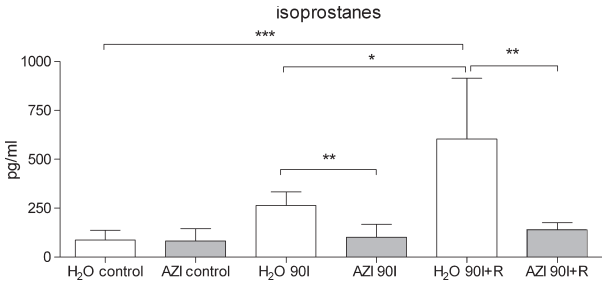

Figure 3 Oxidative stress, measured by 8-isoprostanes, in BAL of the left lung in the different study groups. $*P < 0.05$; $**P < 0.01$; $***P < 0.005$. I, ischaemia; R, reperfusion; AZI, azithromycin.

water-treated animals show significantly more neutrophils than the AZI-treated animals (Fig. 4e and f).

Discussion

In this study, we found that numbers of leukocytes, lymphocytes and neutrophils as well as IL-1β levels in BAL after warm ischaemia and reperfusion were significantly lower in animals that were pretreated with AZI than in animals pretreated with H_2O. Isoprostane levels were also lower in animals treated with AZI than in H_2O-treated animals, after ischaemia as well as after ischaemia and reperfusion. A significant positive correlation was seen in the reperfusion groups between 8-isoprostane levels and the number of neutrophils, IL-6, KC and IL-1β levels. These results indicate that oxidative stress is closely related to neutrophilic influx in the airway and to the expression of IL-6, KC and IL-1β. These findings may open new perspectives for donor pretreatment with AZI in the prevention of IRI after LTx.

Azithromycin, an azalide antimicrobial agent, readily penetrates phagocytic cells, making it useful in the treatment of infections caused by intracellular pathogens [16]. In recent years, a variety of reports have been published demonstrating different anti-inflammatory effects of AZI: inhibition of extracellular release of lysosomal enzymes [17], induction of neutrophil apoptosis [18], reduction of pro-inflammatory cytokine levels [19] and of neutrophilia [10]. AZI is structurally different from other macrolides in that it has a 15-membered ring rather than a 14-membered ring. This structural modification allows for improved potency against gram-negative microorganisms, while it retains the effect of compounds with 14-member rings against gram-positive organisms. In addition, the pharmacokinetic profile of this agent is somewhat unique, with a longer half-life and greater distribution into tissues and fluids than other macrolides [20].

In our murine model, AZI was administered in subtherapeutic/subantibiotic doses. Effects of this drug on infections in our model could therefore be excluded.

Previous studies have already demonstrated that AZI concentration in tissue correlates with the presence of inflammation [21]. Our findings in the reperfusion groups are in accordance with these results. Isoprostane levels were significantly reduced after ischaemia in the AZI pretreated group. In a previous study, we observed that lymphocytes invade the alveolar space already during the ischaemic period (see also Fig. 1c: ischaemic groups compared to controls) [15]. We concluded that inflammation, accompanied by an increase in IL-1β, had already started during the ischaemic period. The high levels of isoprostane in the [H_2O 90I] group in this study confirms this hypothesis as inflammation is accompanied by oxidative stress. Al-Mehdi *et al.* [22] indicated that the generation of oxidizing species in the lungs is the result of IRI. AZI has been shown to act as a free radical scavenger by lowering 8-isoprostane levels *in vitro* [14]. We

Table 1. Correlation analysis.

BAL	Leu	Mac	Lym	Neu	8-Iso	IL-6	IL-1β	KC	GM-CSF	TNF-α	MCP-1
Leu	x	0.85***	0.79**	0.73**	0.57	0.60*	0.82**	0.69*	−0.05	−0.15	0.20
Mac		x	0.55	0.35	0.29	0.42	0.63*	0.51	0.14	0.064	0.39
Lym			x	0.62*	0.48	0.61*	0.85***	−0.20	0.019	−0.072	0.21
Neu				x	0.72**	0.52	0.52	0.027	−0.22	−0.31	−0.22
8-iso					x	0.84***	0.62*	0.88***	0.31	0.14	0.20
IL-6						x	0.65*	0.88**	0.37	0.17	0.45
IL-1β							x	0.72**	0.28	0.12	0.32
KC								x	0.34	0.24	0.32
GM-CSF									x	0.89***	0.27
TNF-α										x	0.0032
MCP-1											x

Leu, leukocytes; Mac, macrophages; Lym, lymphocytes; Neu, neutrophils.
$*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

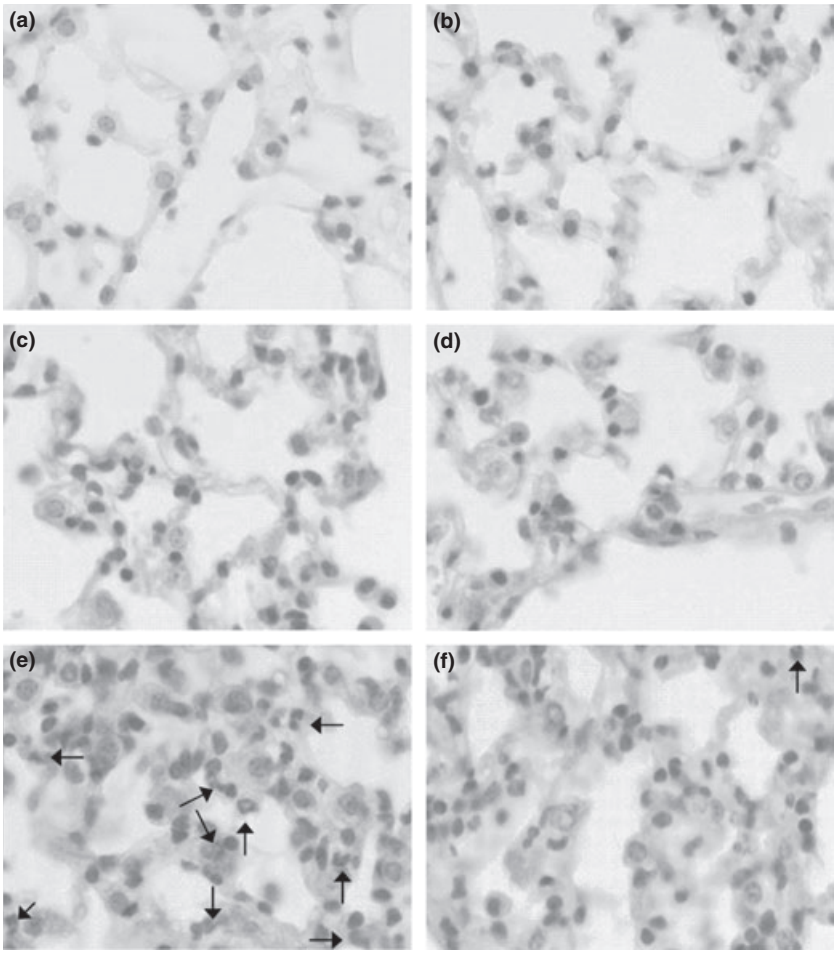

Figure 4 Haematoxylin and eosin stained lung biopsies (1000x) in H_2O control (a), AZI control (b), H_2O 90I (c), AZI 90I (d), H_2O 90I + R (e) and AZI 90I + R (f). An important increase in neutrophils was seen in [H_2O 90I + R]. AZI treatment reduced this influx. Neutrophils are pointed out (arrow). I, ischaemia; R, reperfusion.

demonstrated in this study that AZI is able to reduce oxidative stress occurring during ischaemia but obviously needs more time to create an effect on cells and pro-inflammatory cytokines (after reperfusion).

In the reperfusion groups, AZI treatment had a significant effect on lymphocyte and neutrophil numbers, together with IL-1β and isoprostane levels. In these groups, the numbers of neutrophils correlated positively with oxidative stress. Our group has previously demonstrated that this macrolide antibiotic reduces neutrophilia and IL-8 mRNA in patients suffering from BOS after LTx [10]. In this study, there was a decrease in KC levels, the murine homologue of human GRO-α (Fig. 2b), another neutrophils chemo-attractant, although not significant ($P = 0.25$). A significant difference might be seen when mRNA was analyzed instead of protein. Until now, a murine homologue of IL-8 has not been found. Tamaoki *et al.* [23] recently demonstrated that IL-1 levels were significantly decreased in their model of diffuse panbronchiolitis, possibly through NF-κB or AP-1, although this needs further investigation. IL-6 levels in this study were not significantly different between the reperfusion groups treated with H_2O or AZI. Other investigators, on the other hand, demonstrated lower IL-6 levels after treatment with AZI [19]. The difference with findings from our study might be explained by the fact that this cytokine was measured too early. There was no effect on the number of macrophages in our model, confirming the results of Tsai *et al.* [24]. Lymphocyte numbers were significantly reduced.

In this study, two control groups were included to investigate the effect of AZI on normal lungs. No impact of AZI was found for all measured parameters. A limitation of this study is that the animals in these groups were not ventilated for a long period, as BAL was performed immediately after thoracotomy. However, we have previously demonstrated that there was no difference in BAL inflammatory parameters between groups with a short or long ventilation period after thoracotomy [15]. Gavage itself also did not influence our results as data from this study are comparable with data in sham animals without gavage [15].

In this study, the same treatment regimen (dose, administration route and days) was used as in patients treated for BOS after LTx. Our data now suggest that AZI also has an effect on IRI after LTx. Pretreatment for 14 days, as is used in our murine model, however, is clinically not possible in deceased donors. Recently, Rios *et al.* [25] demonstrated that 1 single high dose of this macrolide antibiotic for the treatment of pneumonia is as effective as a treatment with the same dose, divided over 5 days Further studies are therefore needed to investigate whether a single dose is also effective against IRI after LTx. Also, oral intake is not possible in ventilated donors. Another route to administer AZI (such as aerosol) needs to be further investigated. Hickey *et al.* [26] already showed that inhaled AZI therapy is also effective.

The use of a murine IRI model includes some disadvantages. First, functional data are difficult to obtain. Second, the time of postreperfusion is not that long because it is very difficult to keep these small animals alive for more than 4 h. However, previous studies have shown that after 4 h, a large neutrophil influx is seen in the alveoli [4]. These neutrophils are important for the inflammation during IRI. We therefore think that longer reperfusion periods are not necessary as AZI has already an effect on these cells after 4 h of reperfusion.

To our knowledge, our group is the first one to demonstrate that the macrolide antibiotic AZI has a protective effect on IRI, by reducing lymphocyte and neutrophils numbers, IL-1β levels and oxidative stress. Donor pretreatment with AZI could be a promising method to reduce IRI after LTx.

Authorship

NG: performed research/study, collected data, analyzed data and wrote the paper. LT: performed research/study and collected data. HV: performed research/study. CVDW: designed research/study. BV: designed research/study and wrote the paper. EV: analyzed data. GV: designed research/study. RV, TL and DVR: wrote the paper.

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