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Original Article

Ciprofloxacin plus erythromycin or ambroxol ameliorates endotracheal tube-associated *Pseudomonas aeruginosa* biofilms in a rat model

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ABSTRACT

Background and objective: Pseudomonas aeruginosa is a multi-drug resistant bacterium, with its biofilm-growing mucoid (alginate-producing) strains being particularly resistant. As atomized drug administration is a common practice in pediatric patients, we compared the effect of inhalational therapy with erythromycin plus ciprofloxacin, with that of ambroxol plus ciprofloxacin, against biofilm producing strains of *P. aeruginosa.*

Results: Both combined treatment regimens were associated with a significant reduction in bacterial counts in endotracheal (ET) tubes and lungs, as compared to that observed with ambroxol and erythromycin monotherapies ($P < 0.05$). Ciprofloxacin plus ambroxol appeared to have a higher efficacy than ciprofloxacin plus erythromycin, both in lowering bacterial counts ($P < 0.05$) and in disrupting the structural integrity of biofilm. Histopathological changes in the lungs were milder in the two combined treatment groups, as compared to that in groups treated with single drugs.

Conclusion: Erythromycin or ambroxol in combination with ciprofloxacin could eliminate *P. aeruginosa* biofilms. When combined with ciprofloxacin, ambroxol outperformed erythromycin in eradicating *P. aeruginosa* biofilm.

1. Introduction

With the widespread use of mechanical ventilation in newborns, especially in the preemies, the incidence of respiratory tract infection with biofilm-producing micro-organisms has increased in recent years. *Pseudomonas aeruginosa* (*P. aeruginosa*), which belongs to Gram-negative opportunistic pathogen [1], is one of the commonest causes of nosocomial respiratory infections. *P. aeruginosa* is also one of the most common causes of ventilator-associated pneumonia in neonates [2]. These infections are difficult to treat due to the development of a bacterial biofilm, a term that refers to the matrix-enclosed bacterial populations adherent to each other and/or to surfaces or interfaces with a tenacious extracellular matrix mostly made up of polysaccharides and proteins [2,3].

The biofilms serve as a protective sanctuary for bacteria, and allows for diffusion of nutrients and communication between distant layers within the biofilm community [4]. Several studies have demonstrated that biofilms are important for the persistence of chronic rhinosinusitis, pulmonary infections in cystic fibrosis, chronic otitis media, and device-related infections [4].

Ciprofloxacin, a broad-spectrum antibiotic, has a higher efficacy against Gram-negative bacteria as compared to that against gram-positive bacteria. The susceptibility of *P. aeruginosa* to ciprofloxacin has been demonstrated *in vitro* as well as *in vivo*, especially when it is amongst its planktonic brethren [5]. However, the drug is known to have serious side effects in children and its safety in neonates is not yet established. In our previous study (as yet unpublished), we found that inhalational mode of administration of ciprofloxacin was found to be safer in neonates owing to the relatively low concentration achieved in blood.

Abbreviations: CFU, colony-forming units; CLSI, Clinical Laboratory Standard Institute standard method; EPS, extracellular polymeric substances; ETT, endotracheal tube; DPB, diffuse panbronchiolitis; GMD, GDP-mannose dehydrogenase; LB, Luria broth; IL, interleukin; LPS, lipopolysaccharide; MIC, minimum inhibitory concentration; PA, *Pseudomonas aeruginosa*; PS, pulmonary surfactant; SD, Sprague-Dawley; SEM, scanning electron microscopy; TSB, trypticase soy broth; TNF, tumor necrosis factor; VAP, ventilator associated pneumonia.

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Ambroxol (2-amino-3,5-dibromo-N-[trans-4-hydroxycyclohexyl] benzylamine) is a mucolytic agent that is known to have antioxidant and anti-inflammatory effect in patients with pulmonary infection [6]. Recent reports suggest that ambroxol also promotes the permeability of ciprofloxacin across *P. aeruginosa* biofilms and reduces the volume of extracellular polymeric substances (EPS) [5]. Giovanna et al. found that ambroxol can help alleviate voriconazole resistance of *Candida parapsilosis* biofilm [7]. According to Li et al., ambroxol treatment appeared to disrupt the integrity of the biofilm on the intubation equipment and decrease the bacterial load [8].

Erythromycin, a macrolide, has proved effective in patients with infected cystic fibrosis and Pseudomonas biofilm disease [9]. Towako et al. also demonstrated that the clinical efficacy of erythromycin in diffuse panbronchiolitis (DPB) may be due, at least in part, to the reduction in *P. aeruginosa* biofilm formation [10]. Moreover, erythromycin is bactericidal for *P. aeruginosa*, especially on prolonged exposure [11].

Since both ambroxol and erythromycin appear to counter the effect of biofilms, and since ciprofloxacin administered by inhalational route does not influence its concentration in the blood, we hypothesized that nebulized ciprofloxacin combined with ambroxol or erythromycin will have a superior efficacy in treating *P. aeruginosa* infection.

In the present study, we examined the effect of combined treatment on acute lung infections caused by *P. aeruginosa* with biofilm formation in an endotracheal intubation rat model, and compared the efficacy of the two combination treatments.

2. Materials and methods

2.1. Bacterial strains and their culture

A mucoid strain of *P. aeruginosa* O1 (PAO1), maintained at −70 °C, was obtained from the West China School of Medicine, Sichuan University. This bacterial strain was precultivated in 20% Luria broth (LB), diluted 1:5, and grown overnight at 37 °C in a neutral pH. The bacteria was suspended in saline, centrifugated (3000 g, 4 °C, 10 min) and harvested, resuspended in sterile saline, and fixed to 10^9 colony-forming units (CFU)/mL, as estimated by turbidimetry. Stock cultures were maintained at −70 °C in 30% glycerol.

2.2. Animals

Male and female Sprague-Dawley (SD) (approximately 7-week-old, weighing 200–220 g), rats were purchased from Chongqing Tengxin Biological Technology Limited Company. All rats were housed in a pathogen-free environment and received unlimited sterile food and water in the Laboratory Animal Center at the Children's Hospital of Chongqing Medical University (Chongqing, China). The experimental protocol was approved by the Animal Care and Use Committee at the Chongqing Medical University.

2.3. Erythromycin and ciprofloxacin susceptibility and MIC culture

Minimum inhibitory concentrations (MICs) were determined using the broth microdilution method according to the Clinical Laboratory Standard Institute standard method (CLSI, 2005) [12]. Strains were grown either in trypticase soy broth (TSB; Difco) containing MIC of the antibiotics or in antibiotic-free medium for 48 h at 37 °C.

2.4. Preparation of tubes covered with bacteria biofilms

PAO1 was cultured on a Luria-Bertani medium. Intubation tubes with 3.0 mm diameter, were cut to 1.0 cm lengths, and immersed in 1 mL of 0.5 McIntosh turbidimetric concentration (approximately 1.5×10^8 mL^{-1}), bacterium-saline suspensions for 3 days at 37 °C. Biofilm was formed on the inner surface of these inoculation tubes.

2.5. Experimental model of endotracheal tube precoated with biofilms and drug administration

Before the rats were intubated, the tubes were thoroughly rinsed with 3 mL sterile saline to remove any planktonic bacteria, and the inner fluid was sucked to keep the airway unobstructed. Rats were weighed and anesthetized with a subcutaneous injection of 0.3 mL/g of chloral hydrate. Intratracheal placement and fixation of tubes was done by tracheotomy. After intubation, rats were allowed to recover from anesthesia, eat, and drink spontaneously. Ambroxol was obtained from Boehringer Ingelheim (Shanghai, China), and erythromycin was purchased from Dalian Meiluo (Dalian, China). Rats were housed in a closed chamber (length × width × height: 40 × 30 × 34 cm). An ultrasonic nebulizer (BOY SX; PARI, Starnberg, Germany) was used to deliver an aerosol output at a rate of 6 mL/min [13]. Saline, ambroxol, erythromycin were nebulized respectively. When used in combination, ciprofloxacin was nebulized first and immediately followed by ambroxol or erythromycin. Biofilm-covered intubation model was established in 40 SD rats and these were randomly divided into 5 groups and were administered sterile saline (control); 1 MIC erythromycin; ambroxol (1.07 mg/mL); and 1 MIC erythromycin plus 8 MIC ciprofloxacin; and ambroxol (1.07 mg/mL) plus 8 MIC ciprofloxacin, respectively. Each of the rats was administered the assigned treatment once a day for 7 days.

2.6. Bacteriological and histopathological examination

After one-week treatment, all rats were sacrificed by injecting 0.3 mL/kg of 10% chloral hydrate, and the left and right lungs were excised separately. For bacteriological examination, the right lungs were homogenized, including the implanted tube, and cultured quantitatively. Bacterial enumeration was performed by serially diluting samples on Mueller-Hinton II agar plates, incubating the

Table 1
The bacteria counts in infected lungs.

Groups	Bacteria counts (CFU/ml, $\bar{X} \pm S$)	t	P
Saline	139.250 ± 42.0162	1.825[a]	0.089
Ambroxol	101.625 ± 40.4190	−0.431[b]	0.673
Erythromycin	109.625 ± 33.4747	1.560[c]	0.142
Ery + Cipro	57.750 ± 37.8295		
Amb + Cipro	22.250 ± 17.3184	2.431[d]	0.037

Ery, erythromycin, Amb, ambroxol, Cipro, ciprofloxacin.
[a] Comparison between saline group and ambroxol group.
[b] Comparison between ambroxol group and erythromycin group.
[c] Comparison between saline group and erythromycin group.
[d] Erythromycin plus ciprofloxacin group compared with ambroxol plus ciprofloxacin group.
The t value of saline group compared with erythromycin combined group and ambroxol combined group respectively were 4.077, and 7.282; P value for saline group compared with erythromycin combined group and ambroxol combined group, respectively, were 0.001, 0.000; the t value of ambroxol group compared with erythromycin combined group and ambroxol combined group respectively were 2.242, 5.106, the P value of ambroxol group compared with erythromycin combined group and ambroxol combined group respectively were 0.042, 0.001; the t value of erythromycin group compared with erythromycin combined group and ambroxol combined group, respectively, were 2.905, 6.557, the P value of erythromycin group compared with erythromycin combined group and ambroxol combined group, respectively, were 0.012 and 0.000.