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Original Article

The eliminating effects of clarithromycin combined with ciprofloxacin on *Pseudomonas aeruginosa* biofilms in the middle ear mucosa of rats

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Abstract: Background and Aim: *Pseudomonas aeruginosa* (PA), one of the major pathogenic bacteria, can cause bacteremia, complicated urinary tract infection, and various chronic respiratory diseases. Furthermore, the double drugs, ciprofloxacin (CPFX) and clarithromycin (CAM) have been suggested as effective agents for resisting PA biofilms. The present study was aimed to confirm the removing effects of CPFX on PA biofilms and the effects of CPFX combined with CAM on eliminating PA biofilms were also observed, through using CPFX and CAM locally in the tympanum of rats. Method: 36 rats were randomly and equally divided into 3 groups as follows: the control group (group A), the CPFX group (group B and the CPFX+CAM group (group C). the right tympanic cavities of all the rats were injected with PA bacterial medium by tympanic puncture, in which way the suppurative otitis media models were created. 10 days later, the right tympanic cavities of all the rats were injected with saline, CPFX liquid and CPFX-CAM for 4 days (once a day), respectively. At the day 1st, 3rd, 8th, 14th after first administration of drugs, 3 rats of each group were sacrificed and tympanic bullas were taken out. After a few steps of treatment, the middle ear mucosa specimens were observed by a scanning electron microscope (SEM). The SEM images were analyzed by the software ImageJ, through which the coverage rates of the bacterial biofilms on the mucosal were obtained. Result: (1) The coverage rates of the bacterial biofilms of group A and B were about 90% on the day 3rd, 8th, 14th and there existed no significant difference between A and B among the three time points. (2) Regarding group C, the coverage rates presented a decreasing trend on the day 3rd, 8th, 14th: 88.11 ± 6.09%, 33.94 ± 13.53% and 19.52 ± 9.34% respectively. Moreover, the distinctions of three pair-wise comparisons were statistically significant ($t_{C3\&C8}$, $t_{C3\&C14}$, $t_{C8\&C14}$ were respectively 14.140, 23.825, 3.397, all $P < 0.01$). (3) On the day 3rd, there was no statistical significance in the coverage rate in group C compared with group A and B. However, on the day 8th and 14th, group C had a smaller coverage rate than group A and B, and the differences between A and B, as well as A and C were statistically significant ($t_{C8\&A8}$, $t_{C8\&B8}$, $t_{C14\&A14}$, $t_{C14\&B14}$ were respectively 15.364, 15.025, 27.257, 27.968, all $P < 0.001$). *Conclusion:* The combined role of CPFX and CAM was significantly positive in eliminating PA-induced biofilms in the middle ear mucosa of rats when CPFX and CAM have been used for more than 8 days, while CPFX alone was not that effective.

Keywords: *Pseudomonas aeruginosa* (PA), bacterial biofilms, rats models, clarithromycin (CAM), ciprofloxacin (CPFX)

Introduction

Pseudomonas aeruginosa (PA) is a major pathogen of chronic suppurative otitis media (CSOM) [1] and there exist biofilms in the middle ear mucosa in CSOM caused by PA [2]. A major reason for its prominence as a pathogen is its high intrinsic resistance to antibiotics [3]. Fluoroquinolone and macrolides are widely used for the treatment of PA because they show broad activity against organisms isolation, which contribute to the effect of antibiotics [4]. Feng suggested that the combination of them can be one of the therapeutic manners against biofilm bacteria and it has been proved in several vitro experiments that the combination therapy of CPFX and CAM [5], two corresponding representative drugs of antibiotics, is one of the most effective treatments for PA biofilms [6]. The mechanisms are displayed as fol-

lows: (1) Fluoroquinolone could inhibit DNA synthesis and duplication of bacteria by means of resisting DNA gyrase [7]. Nonetheless, fluoroquinolone alone is not effective enough to eliminate the formation of PA biofilms, which might be mainly due to that it fails to pass through the extracellular matrix to remove the bacteria inside the biofilms. (2) 14, 15-membered macrolides can curb the activity of guanosine diphosphate mannose dehydrogenase in the synthesis pathway of alginate, which is the main component of exopolysaccharides (EPS) protein complex of PA biofilms [8].

Until now, however, there have been few studies reporting the effects of antibiotics on eliminating biofilms formed inside the animals' body. Previously, our group had found that the biofilms, formed in the middle ear mucosa of rats suffering from PA-induced CSOM, became mature and stable after 10 days of post infection. In the present study, the in vitro mechanism of eliminating biofilms with antibiotics were applied in the CSOM rat model, through which the role of fluoroquinolone alone and the combined role of fluoroquinolone and macrolides in eliminating PA biofilms were observed, respectively, providing experimental support for drug selection in treating CSOM clinically.

Material and methods

Experiment material

Animals and their grouping: 36 Wistar rats of either sex, with the body mass of approximately 200-300 g, were taken from the animal department of Tongji Medical College, Huazhong University of Science and Technology. All the rats were sensitive to auricle reflection. Since electro-otoscopy examination required complete tympanic membrane and clear light cone, the existence of infection of external auditory canal, tympanic membrane, middle ear and likewise should be excluded. 36 rats were divided into 3 groups according to the random number table: the control group (group A), the CPFX group (group B) and the CPFX+CAM combination group (group C). All the rats were raised in clean and quiet environments separately.

Bacterial strain

PA, numbered as CCTCC-AB91095, was provided by Department of Microbiology, Tongji Medical College, Huazhong University of Science and Technology. And it was then made into bacterial medium with a concentration of 1 × 106 CFU·ml^{-1}.

Main reagents

CPFX (batch number: 130451-200302) and CAM (batch number: 130558-200902) standards were purchased from National Institutes for Food and Drug Control.

Main instruments

FEI quanta 200-type Scanning Electron Microscope (produced by Dutch FEI company).

The construction of suppurative otitis media rat model

All the rats were given general anesthesia with 10% chloral hydrate, ip. 350 mg·kg^{-1}, and the right external auditory canals were disinfected with 75% ethanol then. 1 ml sterilized syringe was used to take 100 mL PA bacterial medium with a concentration of 1 × 106 CFU·mL^{-1} and then the 27 G lumbar puncture needle was connected. With the help of optical fiber electro-otoscope, the PA bacterial medium was injected into tympanum by passing through the posterior and inferior quadrant of pars tensa of tympanic membrane. All rats were left intact within 10 days.

Drug administration to tympanum through tympanic membrane

Drug preparation: CPFX and CAM standards were diluted into 0.2% with saline. The drugs used in group C was the mix of 0.2% CPFX and 0.2% CAM, with the volume ratio being 1:1.

Drug administration to tympanum

After bacterial inoculation for 10 days, rats were anesthetized as above and the right external auditory canals were disinfected with 75% ethanol then. The tympanum in group A, group B, and group C were injected with a mixture [100 mL saline, 100 mL 0.2% CPFX, and 100 mL 0.2% CPFX+0.2% CAM (1:1)] separately through the tympanocentesis once a day. At day 1, day 3, day 8 and day 14 after first administration of drugs, 3 rats of each group were put to death under general anesthesia to get biopsies, and no drug was given to the rats. The left rats in each group were given the same treatment as above.

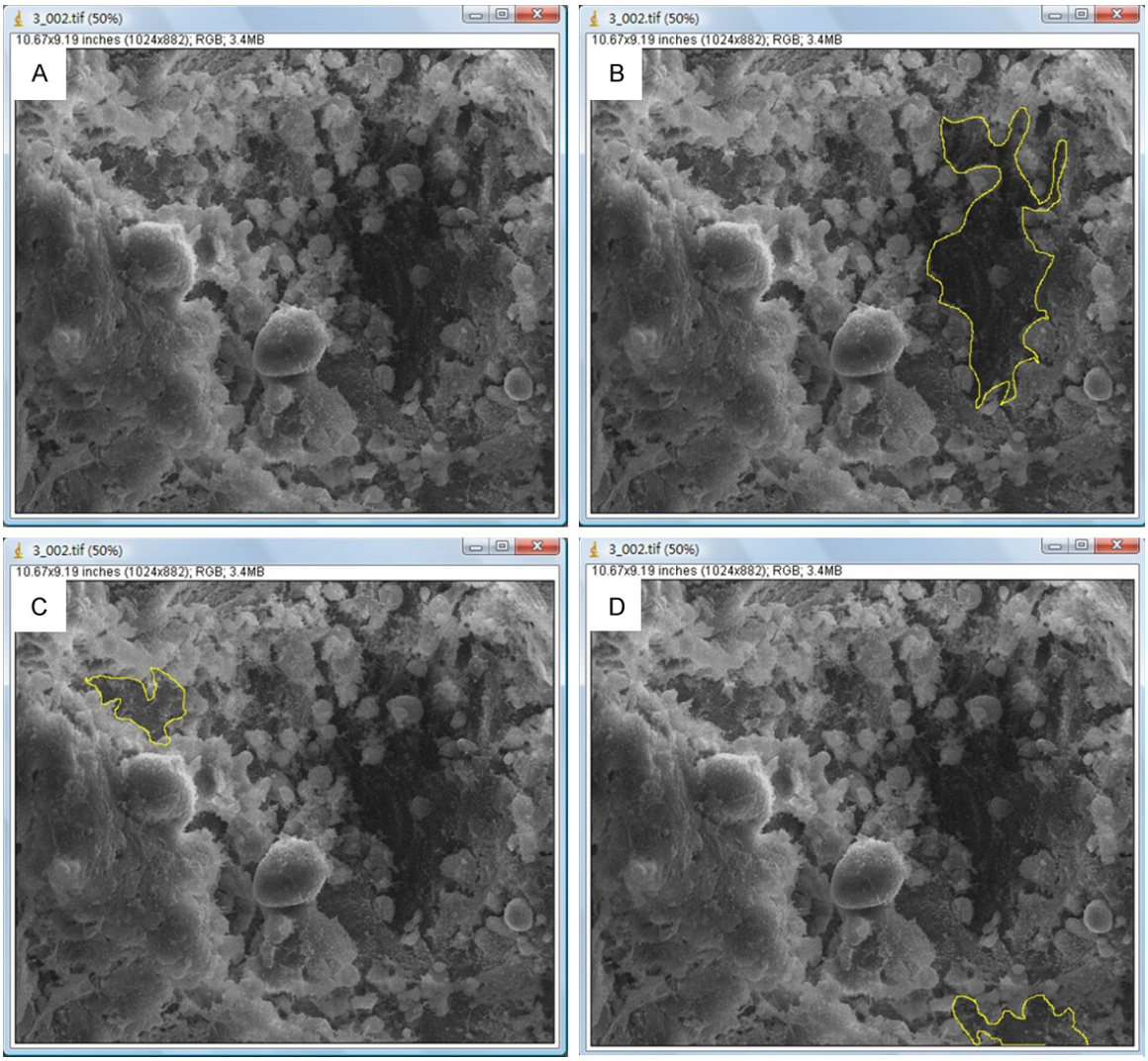

Figure 1. The method to analyze the coverage rate of biofilms for SEM images. The coverage rate of biofilms in the given image can be calculated as (98-7.631-1.438-1.574)/98*100% = 89.14%.

Sample and dispose the middle ear biopsies

Sample biopsies of middle ear mucosa: After anesthesia, the rats were killed by cervical dislocation. The auditory vesicles were sampled and then were cut into upper part and lower part under microscope (i.e. dorsal and ventral). Studies have shown that the upper part of middle ear cavity was easier to form biofilms than the lower part [9]. Therefore, we all sampled the upper part of auditory vesicles.

Disposal of SEM biopsies: The mucosal surface of auditory vesicles was washed with phosphate buffered saline (PBS) at first, and then it was fixed in 2.5% glutaraldehyde PBS, deposited in 4°C freezer for 24 h. In addition, the biopsies were washed with PBS for three times (15 mins each time) and then were fixed in 1% osmium tetroxide at room temperature for 1 h; after that, the biopsies were dehydrated with ethanol for 15 mins in different concentrations, from 50%, 70%, 85%, 95%, to 100%; the biopsies were dried by using the critical point dryer, which was mounted on aluminum stubs and coated by gold. Finally, the biopsies were sent to Optoelectronic Technology Lab, Huazhong University of Science and Technology for FEI quanta 200-type SEM observation.

Results evaluation

Observation of biofilm formation with SEM: Each sample was given scans with multiple magnifications in multiple areas. The samples would be considered with bacterial biofilms formation when satisfying the following three

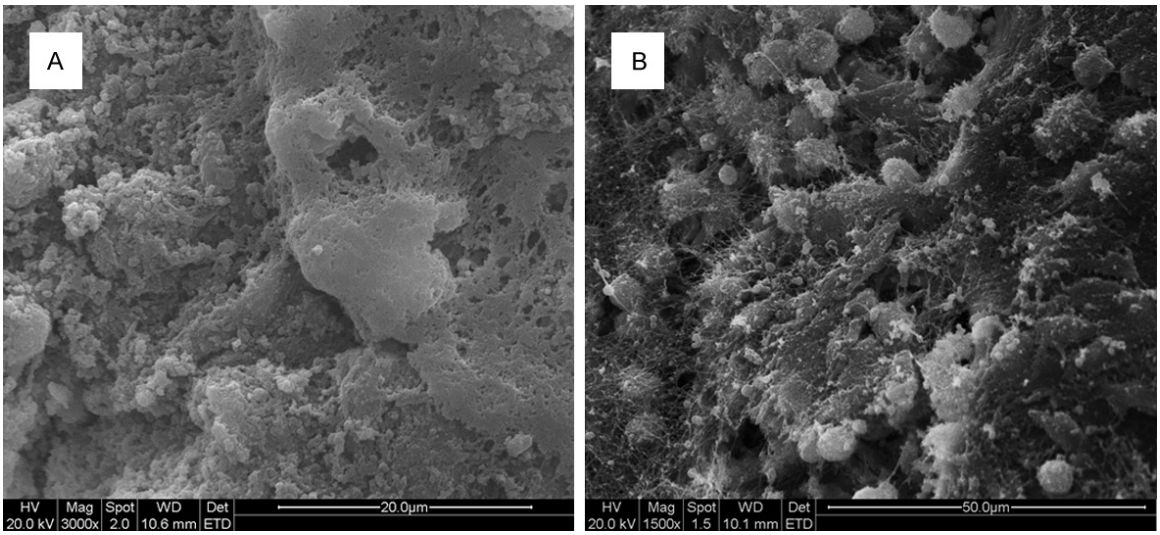

Figure 2. The SEM images of group A at the day 8th and 14th. A. The day 8th. B. The day 14th. Bacterial biofilms were covered with the mucosal surface everywhere, and almost no normal mucosa can be seen.

requirements [10]: (1) There was substance with bacteria-like size and shape; (2) There was amorphous substance surrounding the bacteria; (3) The above substances adhered to the mucosal surface.

The effect evaluation of drug administration

We found that the area with biofilm formation was easily identified when observing the middle ear mucosal SEM images. Each sample at 1000 × magnification in 5 different visual fields was scanned, and the images were saved as TIFF format and analyzed by ImageJ software, through which the coverage rate of biofilm on middle ear mucosa would be obtained. The analyzing process was showed as **Figure 1**.

Statistical method

All the data were analyzed by SPSS11.0. If the data met normal distribution, they would be indicated as $\bar{x} \pm s$. t test was used for comparison among groups, and there was significant difference when $P < 0.05$.

Result

Qualitative evaluation

There were high-density biofilms covering the mucosal surface at each day in both group A and B. In group C, the coverage rate obviously decreased at day 8 and day 14 (**Figures 2** and **3**).

Quantitative evaluation

ImageJ software was performed to quantitatively analyze the SEM images at day 3, day 8 and day 14 in each group of rats, and the results were shown as follows:

In group A, in group B, and comparison between group A and B: The coverage rates of the bacterial biofilms of group A and B remained about 90% on the day 3rd, 8th, 14th with no statistical significance and no significant distinction was present between group A and B in each of the three time points (**Table 1**).

In group C, and comparison between group C and group A, B: The coverage rates in group C at day 3, day 8 and day 14 were respectively 88.11 ± 6.09%, 33.94 ± 13.53% and 19.52 ± 9.34%, which could be expressed as a decreasing trend. The differences of three pair-wise comparisons were statistically significant ($t_{C3\&C8}$, $t_{C3\&C14}$, $t_{C8\&C14}$ were respectively 14.140, 23.825, 3.397, all P < 0. 01). On the day 3rd, there was no statistical significance in the coverage rate when comparing group C with group A and B. On the day 8th and 14th, group C had a smaller coverage rate than group A and B at the same time points, and the differences between A and B, A and C were statistically significant ($t_{C8\&A8}$, $t_{C8\&B8}$, $t_{C14\&A14}$, $t_{C14\&B14}$ were respectively 15.364, 15.025, 27.257, 27.968, all P < 0.001).

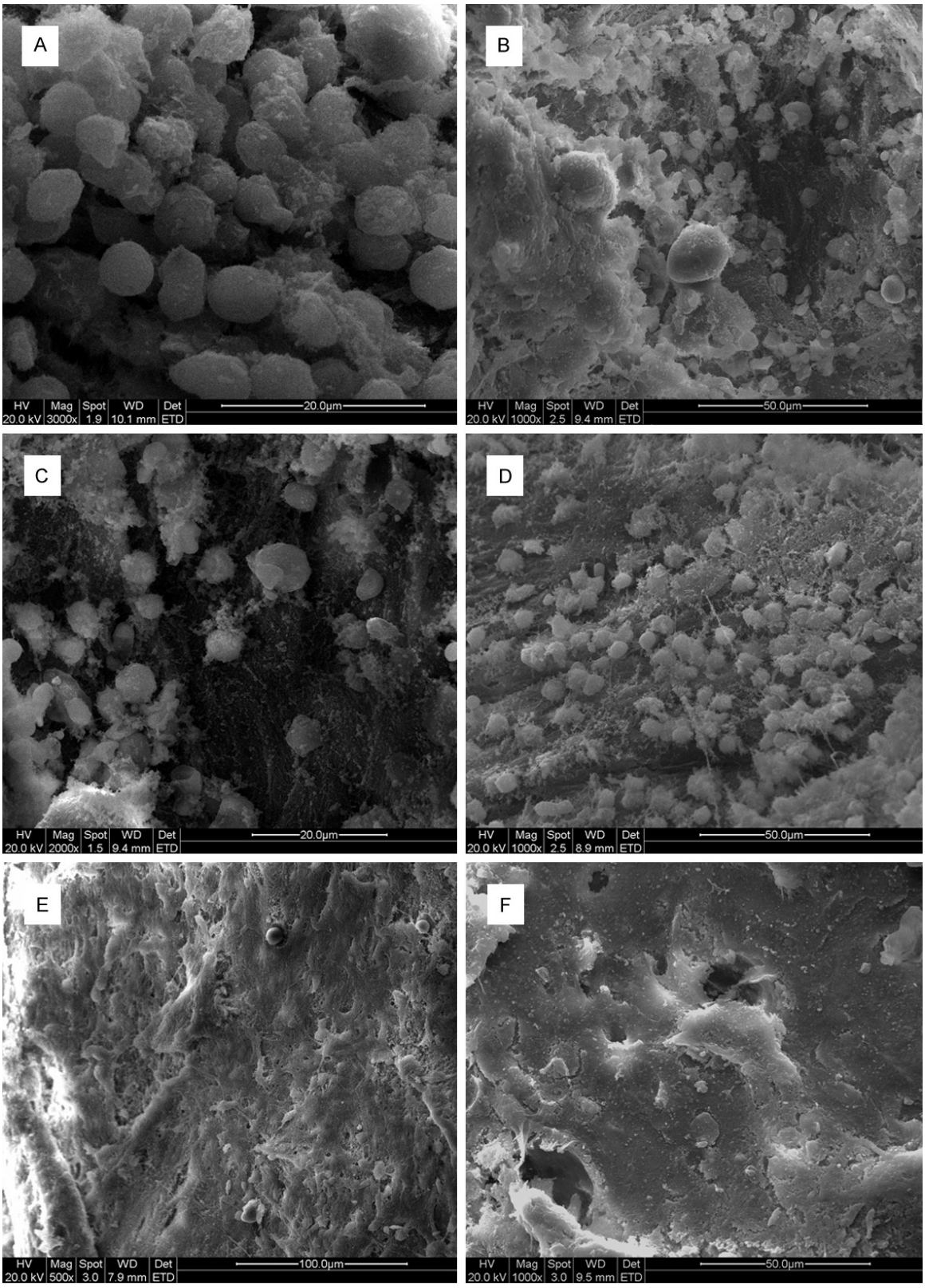

Figure 3. The SEM images of group B and C at each time point. A-C. Most part of mucosa was covered with the mushroom-shaped biofilms at the day 3rd, 8th and 14th in group B. D. Most part of mucosa was covered with biofilms at the day 3rd in group C. E. Only few mushroom-shaped structures can be seen at the day 8th in group C. F. It was difficult to see the typical biofilms structure at the day 14th in group C.

Table 1. Biofilms coverage rates of each group at each time point

Group	Day 3	Day 8	Day 14	Day 3 vs. Day 8	Day 3 vs. Day 14	Day 8 vs. Day 14
A	90.03 ± 6.23	90.89 ± 4.80	92.21 ± 4.41	$P > 0.05$	$P > 0.05$	$P > 0.05$
B	90.52 ± 4.21	90.57 ± 5.48	92.61 ± 4.79	$P > 0.05$	$P > 0.05$	$P > 0.05$
C	88.11 ± 6.09	33.94 ± 13.53	19.52 ± 9.34	$P < 0.01$	$P < 0.01$	$P < 0.01$
C vs. A	$P > 0.05$	$P < 0.001$	$P < 0.001$	-	-	-
C vs. B	$P > 0.05$	$P < 0.001$	$P < 0.001$	-	-	-

Discussion

According to a public announcement by the US National Institutes of Health, "Biofilms are medically important, accounting for over 80% of microbial infections in the body". It is composed of bacterium body, exopolysaccharides, water and bacterial secretory products. Furthermore, the bacterial population was surrounded by its own secretory polysaccharide matrix, forming a highly organized heterogeneous structure. Klausen expressly performed an investigation with time-lapse confocal laser scanning microscopy (CLSM) of biofilms formed by various combinations of color-coded PA wild type and motility mutants to study bacterial migration patterns during PA biofilm development [11]. Davies examined the mechanisms that underlie biofilm resistance to antimicrobial therapy [12], they concluded that the character of the biofilm structure makes the biofilm bacteria naturally resistant to antibiotics, and the key issue is that the extracellular matrix of biofilm blocks the penetration of antibiotics, making it tough for antibiotics to eliminate the bacteria inside the biofilm and thus the bacterial infection is hard to be cured.

In fact, association studies on CSOM biofilms were rare with animal or human model. Roland summarized that biofilms might be a major cause of CSOM owing to the fact that PA and *Staphylococcus aureus* [1], both of which were skilled in forming biofilms, were the most commonly found pathogens related with CSOM. It was also demonstrated that biofilms were existent in human CSOM patients and the detection rate of middle ear mucosal biofilm in CSOM patients was much pronounced than that in patients with other disorders in the middle ear. In that study [10], SEM and CLSM were utilized and it was found that 6 biofilms were observed in 10 middle ear mucosal biopsies of CSOM patients, while only one biofilm was formed among the 10 biopsies in the control group, indicating that biofilms might play a significant role in causing CSOM.

Another research used PA to infect the middle ear cavity of the primate and biofilm was found in middle ear mucosa at week 1 and week 4 after bacterial infection of the middle ear cavity [2]. PA was believed to have a strong ability to form biofilms as reported that biofilms were formed in PA-induced sinusitis animal model 5 days after infection [13]. As the pathological process of PA infecting middle ear cavity was similar to that of PA infecting nasal sinuses, rats were selected as research objects in this group and the formation of middle ear mucosal biofilm were fortunately observed by SEM and CLSM at the early stage of infection (within 3 weeks). Additionally, biofilm formation was found to begin at the 6^{th} day after bacterial inoculation, and the biofilm at the 10^{th} day was approximately the most representative, which remained stable within 3 weeks.

Studies worldwide about the functional effects of antibiotics on PA biofilms are still limited to in vitro experiments. The antibiotics studied are primarily made up of fluoroquinolone and macrolides antibiotics. Among the above double drugs, fluoroquinolone has been regarded as the most effective antibiotic for killing bacteria inside biofilm, through inhibiting DNA synthesis and replication of bacteria [7]. However, fluoroquinolone alone could not effectively and efficiently remove the biofilms, which might be due to impermeability of the PA biofilms. As is revealed by the investigators, PA adheres to the surface of tissues, splitting and reproducing constantly, and secrets a lot of Extracellular Polymeric Substances (EPS) protein-complex (mainly alginate) [14], in which way a crucial structural composition of the PA biofilms were, therefore, formed. The PAs lying deep inside EPS would become sensitive to anti-PA drugs only when the structure of biofilms change.

Moreover, 14, 15-membered macrolides has been reported to inhibit the synthesis of alginate via the inhibition of guanosine diphosphate mannose dehydrogenase [8], enhancing the permeability of other sensitive antibiotics into the PA biofilms [15]. To sum up, the collaboration of fluoroquinolone and macrolides could become an ideal treatment for PA biofilms, as reported before by Kumon et al. [6].

However, up to now, the usage of antibiotics in animal models has rarely been reported to observe the eliminating effect of double antibiotics on the already formed PA. In this research, we applied the in vitro mechanism of removing biofilms with antibiotics to a CSOM rat model, and subsequently the effect of fluoroquinolone alone and the effects of fluoroquinolone combined with macrolides on eliminating PA biofilms were examined. Previously, erythromycin and azithromycin were two antibiotics most commonly used for in vitro experiments [16]. Currently, CAM, the new generation of macrolides antibiotics, is advantageous in wide antibacterial spectrum, strong antibacterial activity, robust tissue, high cellular penetration and long half-life, due to which it became one of the most common macrolides antibiotic for in vitro experiments about anti-PA biofilms [17]. Therefore, in our study we used CAM and CPFX as the representative agents of macrolides and fluoroquinolone, respectively. To be added, interesting results could also be drawn that the combination of CPFX and CAM was significantly effective in eliminating biofilms after more than 8 days of treatment, while CPFX alone was not that much effective.

Besides, morphological descriptions by either SEM or CLSM were principally utilized in previous studies [18, 19]. Nonetheless, quantitative data was nearly impossible to be obtained because of the fact that the density of biofilms was only comparable in different images. Instead, Michael performed Carnoy software to analyze the SEM images of the middle ear mucosal of Streptococcus pneumonia-infected chinchilla [20], successfully evaluating the severity of bacterial infection through calculating the coverage rate of biofilm on mucosal surface. In this study, Michael's methods were imitated, and ImageJ software was performed to analyze the SEM images, thus providing quantitative and scientific judging methods to evaluate the effects of antibiotics on eliminating biofilms.

Disclosure of conflict of interest

None.

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