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Effects on the *Pseudomonas aeruginosa* in biofilm of fleroxacin combined with urokinase or earthworm kinase

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ABSTRACT Effects of fleroxacin (FLRX) combined with urokinase (UK) or earthworm kinase (EK) on the bacterial biofilm (BBF) of *Pseudomonas aeruginosa* were investigated. MICs were determined by using microdilution method; bacterial biofilm was defined by a rapid staining procedure of $AgNO_3$; the morphology of biofilm was observed by scanning electron microscopy; viable bacteria were counted by MTT method. The photography of SEM showed that the morphology of biofilm treated by FLRX combined with urokinase (UK) or earthworm xinase (EK) was markedly changed and the counts of viable bacteria in the BBF treated by FLRX plus UK or EK were significantly decreased than with FLRX alone. The effects on the BBF treated by FLRX plus UK or EK were synergic.

KEY WORDS Urokinase; Earthworm kinase; Bacterial biofilm; *Pseudomonas aeruginosa*; Fleroxacin

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1 Introduction

Bacterial biofilm[1] is defined as mucoid matrix-enclosed bacterial populations adhere to each other and to surface. Biofilms have been found on the surfaces of biomaterials and tissues in chronic infectious diseases caused by bacteria that are characterized by resistance to chemotherapy and resistance to clearance by humoral or cellular host defense mechanisms[2]. So some efforts have been made to eradicate biofilm bacteria. In our previous report[3], we presented the new finding that treatment of the biofilms with fleroxacin resulted in the eradication of slime-like structure and bactericidal activity to *Ps. aeruginosa* in biofilm. But the action of fleroxacin on biofilms was partial and middling.

Proteolytic enzymes and protease inhibitors have been shown *in vitro* to have the capacity to depress or inhibit some fundamental activities of bacteria[4]. It is unclear whether the enzymes have activity on bacterial biofilm. In the study we investigated the interaction of fleroxacin combined with urokinase or earthworm kinase on biofilm of *Ps. aeruginosa*.

2 Materials and methods

Organisms

The tested strains were *Ps. aeruginosa* ATCC27853 and 10 strains of clinical isolates. The organisms were identified with VITEK Microbiology System.

Drugs and reagents

Urokinase (White Swan Pharmaceutical Manufactory Co. in Harbin), earthworm kinase (Institute of Aerial Medicine in Beijing), fleroxacin (Kyorin Pharmaceutical Co. in Japan), MTT (Boeringer Mannheim in Germany), Trypticase Soy Broth (TSB, BioMerieux in France), Mueller Hinton Broth (MHB, Difco in USA), Silver nitrate A. R.

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Equipments

Scanning Electron Microscope (SEM Hitachi in Japan), VITEK Microbiology System (BioMerieux), Microplate Reader (3550UV, Bio-Rad), Silicon discs (Beijing Medical Material manufactory).

Procedure

Determination of minimal inhibitory concentration (MIC)

MIC of fleroxacin was measured by microdilution method.

Establishment of bacterial biofilm model[5] The sterile silicon discs (diameter 1cm) were added in wells of a 24-well tissue culture plate and incubated with *Ps. aeruginosa* suspension (1ml) for 7 days. The discs were washed with TSB and fresh TSB was added every 24h, and then TSB containing different concentrations of fleroxacin plus urokinase or earthworm kinase was added for 24h while the steady biofilm was formed.

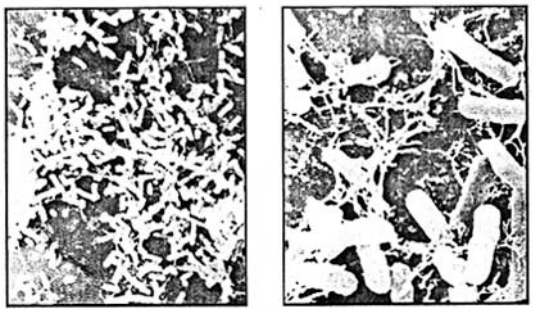
Morphological identification of bacterial biofilm The biofilm was rapidly identified by staining of silver nitrate[6]. The ultrastructure photography of biofilm was observed with SEM[7].

3 Results

MIC of fleroxacin against planktonic *Ps. aeruginosa*

The ranges of MIC, MIC_{50} and MIC_{90} were 1.0～80, 1.0 and 5.64mg/L, respectively.

Morphological observation of bacterial biofilm, see Fig. 1～4.



A　　B

Fig. 1　The SEM photography of untreated bacterial biofilm of *Ps. aeruginosa in vitro* (A and B) The cells of *Ps. aeruginosa* were adhered in microcolonies and surrounded by glycocalex metarials

Fig. 2　The SEM photography of bacterial biofilm of *Ps. aeruginosa in vitro* treated by 4×MIC of fleroxacin
The cells of *Ps aeruginosa* in biofilm and glycocalex materials were reduced

Fig. 3　The SEM photography of bacterial biofilm of *Ps aeruginosa in vitro* treated with 4×MIC of fleroxacin combined with 2500 u/ml of urokinase

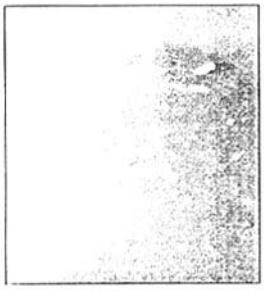


Fig. 4　The SEM photography of bacterial biofilm of *Ps aeruginosa in vitro* treated by 4×MIC of fleroxacin combined with 1250 u/ml of earthworm kinase

There were black materials (glycocalyx) and some viable bacilli in the space using the rapid identification method of silver staining.

The electronphotography of biofilm showed that the cells of *Ps. aeruginosa* were coagulated in microcolonies and surrounded by a thick layer of mucoid glycocalyx materials in control group (untreated). Both the cells of *Ps. aeruginosa* and glycocalyx materials in biofilm treated by 4×MIC of fleroxacin were reduced; but the cells were less and most of them were broken into pieces and there was nearly no mucoid materials in the groups treated by fleroxacin plus urokinase or earthworm kinase.

Viable count of *Ps. aeruginosa* in biofilm, see Tab. 1 and Tab. 2.

Tab. 1 Effects of FLRX combined with UK on the count of viable bacteria (lg CFU/cm²) in bacterial biofilm (n=10)

FLRX (×MIC)	Urokinase (U/ml)				
	0	1250	2500	5000	10000
0	6.78±0.51	6.81±0.50	6.88±0.28	6.42±0.26	6.31±0.45
1/2	6.30±0.60	5.46±0.25*	5.55±0.29*	5.52±0.47*	5.24±0.40*
1	5.34±0.35^	5.19±0.26*#	5.12±0.14*#	5.04±0.19*#	5.00±0.14*#
4	5.25±0.07^	5.18±0.15*#	5.11±0.15*#	5.01±0.21*#	4.80±0.17*#

*: P<0.05 vs. group of 0 U/ml of urokinase; #: P<0.05 vs. group of 0×MIC of fleroxacin;
^: P<0.05 vs. group of blank control.

Tab. 2 Effects of FLRX combined with EK on the count of viable bacteria (lg CFU/cm²) in BBF (n=10)

FLRX (×MIC)	Earthworm kinase(U/ml)				
	0	78	312	1250	5000
0	6.48±0.96	6.27±0.28	5.56±0.83	6.46±0.89	6.14±0.77
1/2	6.21±0.42	5.87±1.11	4.82±1.14*	4.47±1.19*#	3.89±0.87*#
1	5.26±1.37^	4.23±1.50*	3.58±0.49*#	3.54±0.57*#	3.52±0.52*#
4	5.20±0.59^	3.48±0.39*#	3.45±0.54*#	3.42±0.43*#	3.40±0.44*#

*: P<0.05 vs. group of 0 U/ml of earthworm kinase; #: P<0.05 vs. group of 0×MIC of fleroxacin;
^: P<0.05 vs. group of blank control.

Viable count of *Ps. aeruginosa* cells in biofilm treated by fleroxacin plus urokinase or earthworm kinase was significantly less than that treated by fleroxacin alone. The synthetic antibacterial activity of fleroxacin plus earthworm kinase was stronger than plus urokinase (P<0.05).

4 Discussion

The bacterial biofilm associated infections are difficult-treated[8,9]. The infections include the medical biomaterial-related infections and some chronic infections.

It is now well-documented that adherent bacteria are less susceptible to antimicrobial agents than their free floating cells[10]. In particular, some study have shown that resistance to antibiotics of *Ps. aeruginosa* in biofilm was 100-fold greater than their planktonic cells[11]. The mechanism of resistance of bacterial biofilm has get to be elucidated, but a potentially important factor is production of glycocalyx which enable cells growing in biofilm to evade host defences and the activity of antimicrobial agents[12].

The results of the present study suggested that the urokinase or earthworm kinase is able to greatly enhance the activity of fleroxaxin against the sessile *Ps. aeruginosa* cells in biofilm.

The enhancing action of urokinase and earthworm kinase discussed above has not been reported previously, the molecular mechanism is under investigation. It may be associated with that proteolytic enzyme hydrolyze glycocalyx produced by bacteria, inhibit the development of biofilm and destroy the normal structure of

biofilm.

The clinical studies on treatment of biofilm associated infections in humans would verify the effectiveness of a combined schedule of fleroxacin with proteolytic enzyme in both prophylactic and therapeutic practise in future.

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尿激酶或蚓激酶与氟罗沙星联合应用对细菌生物被膜的影响

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摘要: 探讨尿激酶或蚓激酶与氟罗沙星联合应用对铜绿假单胞菌生物被膜的影响。MIC 采用微量稀释法测定,应用银染法快速鉴定细菌生物被膜,应用扫描电镜观察细菌生物被膜中细菌的形态并用 MTT 法测定生物被膜中细菌数。扫描电镜显示通过尿激酶或蚓激酶与氟罗沙星联合应用,细菌生物被膜有显著改变,生物被膜中的细菌数量比单独应用氟罗沙星显著减少。实验结果表明尿激酶或蚓激酶与氟罗沙星联合应用表现出协同效应。

关键词: 尿激酶; 蚓激酶; 细菌生物被膜; 铜绿假单胞菌; 氟罗沙星