

World Journal of *Clinical Cases*

World J Clin Cases 2023 June 16; 11(17): 3932-4209



REVIEW

- 3932 Liver replacement therapy with extracorporeal blood purification techniques current knowledge and future directions

Papamichalis P, Oikonomou KG, Valsamaki A, Xanthoudaki M, Katsiafylloudis P, Papapostolou E, Skoura AL, Papamichalis M, Karvouniaris M, Koutras A, Vaitis E, Sarchosi S, Papadogoulas A, Papadopoulos D

MINIREVIEWS

- 3949 Prediction models for recurrence in patients with small bowel bleeding
Kim JH, Nam SJ
- 3958 Investigation of possible relationship between atopic dermatitis and salivary biomarkers, stress, and sleep disorders
Estefan J, Ferreira DC, Cavalcante FS, dos Santos KRN, Ribeiro M
- 3967 Value of clinical applications of differential pressure and relative pressure imaging in the left ventricle
Zheng AS, Yu HX
- 3976 Low-dose immunotherapy as a potentiator to increase the response with neo-adjuvant chemotherapy in oral cancers
Rathinasamy N, Muthu S, Krishnan A
- 3980 Kidney disease in patients with chronic liver disease: Does sex matter?
Cooper KM, Colletta A, Moulton K, Ralton KM, Devuni D

ORIGINAL ARTICLE

Case Control Study

- 3993 Elabela is a reliable biomarker for predicting early onset preeclampsia: A comparative study
Amer Ali E, Nori W, Salman AF, Al-Rawi TSS, Hameed BH, Al-Ani RM

Retrospective Cohort Study

- 4003 Acute-on-chronic liver failure is independently associated with higher mortality for cirrhotic patients with acute esophageal variceal hemorrhage: Retrospective cohort study
Terres AZ, Balbinot RS, Muscope ALF, Longen ML, Schena B, Cini BT, Rost Jr GL, Balensiefer JIL, Eberhardt LZ, Balbinot RA, Balbinot SS, Soldara J

Retrospective Study

- 4019 Elastic fiber degradation in the development of pediatric granuloma annulare: Report of 39 cases
Zhang DY, Zhang L, Yang QY, Xie YC, Jiang HC, Li JZ, Shu H

- 4026** Anti-bacterial mechanism of baicalin-tobramycin combination on carbapenem-resistant *Pseudomonas aeruginosa*

Jin LM, Shen H, Che XY, Jin Y, Yuan CM, Zhang NH

SYSTEMATIC REVIEWS

- 4035** Acknowledging the use of botanicals to treat diabetic foot ulcer during the 21st century: A systematic review

Narzary I, Swarnakar A, Kalita M, Middha SK, Usha T, Babu D, Mochahary B, Brahma S, Basumatary J, Goyal AK

CASE REPORT

- 4060** Pregabalin induced balance disorder, asthenia, edema, and constipation in an elderly adult: A case report

Ma LP, Wen C, Zhao TX, Jiang XM, Gu J

- 4065** Emergency internal iliac artery temporary occlusion after massive hemorrhage during surgery of cesarean scar pregnancy: A case report

Xie JP, Chen LL, Lv W, Li W, Fang H, Zhu G

- 4072** Hemophagocytic lymphohistiocytosis after autologous stem cell transplantation in angioimmunoblastic T-cell lymphoma: A case report

Zhang ZR, Dou AX, Liu Y, Zhu HB, Jia HP, Kong QH, Sun LK, Qin AQ

- 4079** Successful reconstruction of an ankle defect with free tissue transfer in a hemophilia A patient with repetitive hemoarthrosis: A case report

Lee DY, Lim S, Eo S, Yoon JS

- 4084** Primary pelvic *Echinococcus granulosus* infection: A case report

Abulaiti Y, Kadi A, Tayier B, Tuergan T, Shalayiadang P, Abulizi A, Ahan A

- 4090** Epstein-Barr virus-induced infection-associated hemophagocytic lymphohistiocytosis with acute liver injury: A case report

Sun FY, Ouyang BQ, Li XX, Zhang T, Feng WT, Han YG

- 4098** Cardiac arrest secondary to pulmonary embolism treated with extracorporeal cardiopulmonary resuscitation: Six case reports

Qiu MS, Deng YJ, Yang X, Shao HQ

- 4105** Flared inflammatory episode transforms advanced myelodysplastic syndrome into aplastic pancytopenia: A case report and literature review

Ju B, Xiu NN, Xu J, Yang XD, Sun XY, Zhao XC

- 4117** Frontal penetrating arrow injury: A case report

Rodríguez-Ramos A, Zapata-Castilleja CA, Treviño-González JL, Palacios-Saucedo GC, Sánchez-Cortés RG, Hinojosa-Amaya LG, Nieto-Sanjuanero A, de la O-Cavazos M

- 4123** Chest wall osteochondroma resection with biologic acellular bovine dermal mesh reconstruction in pediatric hereditary multiple exostoses: A case report and review of literature

Alshehri A

- 4133** Massive pulmonary embolism in Klippel-Trenaunay syndrome after leg raising: A case report
Lo CY, Chen KB, Chen LK, Chiou CS
- 4142** Improved super-elastic Ti-Ni alloy wire intrusion arch for skeletal class II malocclusion combined with deep overbite: A case report
Yang CY, Lin CC, Wang LJ, Chen YH, Yu JH
- 4152** Glucocorticoid pulse therapy in an elderly patient with post-COVID-19 organizing pneumonia: A case report
Park S, Jang Y, Koo SM, Nam BD, Yoon HY
- 4159** Endoscopic and surgical treatment of jejunal gallstone ileus caused by cholecystoduodenal fistula: A case report
Fan WJ, Liu M, Feng XX
- 4168** Application of advanced platelet-rich fibrin for through-and-through bony defect during endodontic surgery: Three case reports and review of the literature
Algahtani FN, Almohareb R, Aljamie M, Alkhunaini N, ALHarthi SS, Barakat R
- 4179** Facial Merkel cell carcinoma in a patient with diabetes and hepatitis B: A case report
Ren MY, Shi YJ, Lu W, Fan SS, Tao XH, Ding Y
- 4187** Pregnancy and lactation-associated osteoporosis with pyogenic spondylitis: A case report
Zhai K, Wang L, Wu AF, Qian Y, Huang WM
- 4194** Hourglass-like constriction of the anterior interosseous nerve in the left forearm: A case report
He R, Yu JL, Jin HL, Ng L, Wang JC, Li X, Gai TT, Zhou Y, Li DP
- 4202** Crohn's disease in human immunodeficiency virus-infected patient: A case report
Vinikaite A, Kurlinkus B, Jasinskaite D, Strainiene S, Buineviciute A, Sadauskaite G, Kiudelis V, Kazenaite E

ABOUT COVER

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AIMS AND SCOPE

The primary aim of *World Journal of Clinical Cases* (WJCC, *World J Clin Cases*) is to provide scholars and readers from various fields of clinical medicine with a platform to publish high-quality clinical research articles and communicate their research findings online.

WJCC mainly publishes articles reporting research results and findings obtained in the field of clinical medicine and covering a wide range of topics, including case control studies, retrospective cohort studies, retrospective studies, clinical trials studies, observational studies, prospective studies, randomized controlled trials, randomized clinical trials, systematic reviews, meta-analysis, and case reports.

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Retrospective Study

Anti-bacterial mechanism of baicalin-tobramycin combination on carbapenem-resistant *Pseudomonas aeruginosa*

Li-Min Jin, Hui Shen, Xing-Ying Che, Ye Jin, Chun-Mei Yuan, Neng-Hua Zhang

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Abstract

BACKGROUND

Pseudomonas aeruginosa (*P. aeruginosa*) is an important cause of nosocomial infections, and contributes to high morbidity and mortality, especially in intensive care units. *P. aeruginosa* is considered a 'critical' category bacterial pathogen by the World Health Organization to encourage an urgent need for research and development of new antibiotics against its infections.

AIM

To investigate the effectiveness of baicalin combined with tobramycin therapy as a potential treatment method for carbapenem-resistant *P. aeruginosa* (CRPA) infections.

METHODS

Polymerase chain reaction (PCR) and RT-PCR were used to detect the expression levels of drug-resistant genes (including *VIM*, *IMP* and *OprD2*) and biofilm-related genes (including *algD*, *pslA* and *lasR*) in CRPA that confer resistance to tobramycin, baicalin and tobramycin combined with baicalin (0, 1/8, 1/4, 1/2 and 1MIC).

RESULTS

There was a correlation between biofilm formation and the expression of biofilm-related genes. In addition, *VIM*, *IMP*, *OprD2*, *algD*, *pslA* and *lasR* that confer biofilm production under different concentrations in CRPA were significantly correlated. The synergistic effect of baicalin combined with tobramycin was a significant down-regulation of *VIM*, *IMP*, *algD*, *pslA* and *lasR*.

CONCLUSION

Baicalin combined with tobramycin therapy can be an effective treatment method for patients with CRPA infection.

Key Words: Baicalin; *Pseudomonas aeruginosa*; Tobramycin; Carbapenem-resistant *Pseudomonas aeruginosa*; Therapy

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Core Tip: Baicalin combined with tobramycin therapy shows potential as an effective treatment method for patients with carbapenem-resistant *Pseudomonas aeruginosa* infection, as it significantly down-regulates drug-resistant and biofilm-related genes.

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INTRODUCTION

Pseudomonas aeruginosa (*P. aeruginosa*) is an important cause of nosocomial infections, and contributes to high morbidity and mortality, especially in intensive care units[1,2]. *P. aeruginosa* is considered a 'critical' category bacterial pathogen by the World Health Organization (WHO) to encourage an urgent need for research and development of new antibiotics against its infections. It is an opportunistic pathogen that naturally exists in the human skin, respiratory tract, and gastrointestinal tract. As *P. aeruginosa* is a common cause of respiratory and urinary tract infections, as well as bloodstream infections, its infections are priority healthcare-associated infections in some hospitals. According to data collected by the Global Drug Resistance Surveillance Network from 1996 to 2016, *P. aeruginosa* bloodstream infections accounted for 5.3% of all bloodstream infections in more than 200 medical centers in 45 countries.

With the widespread and irrational use of carbapenem antibiotics to treat *P. aeruginosa* infections, carbapenem-resistant *P. aeruginosa* (CRPA) strains have gradually appeared, presenting a new challenge to existing clinical treatments. In 2017, WHO listed CRPA as one of the three major bacteria requiring urgent development of new drugs to control infection[3]. Although aminoglycoside antibacterial drug-tobramycin has shown strong efficacy against CRPA, they are dose-dependent, have greater renal and ear toxicity, and are prone to drug resistance[4,5]. At the same time, recent research has increasingly focused on the antibacterial effect of compounds found in traditional Chinese medicine. Among them, baicalin has been shown to have a strong effect on *P. aeruginosa* in some studies[6].

In vivo experiments showed that baicalin (100 mg/kg) could reduce mortality in mice infected with drug-resistant *P. aeruginosa*, indicating its potent bacteriostatic effect on this strain[7,8]. Therefore, in this study, the anti-bacterial effect of baicalin combined with tobramycin on CRPA infection was investigated, including the potential synergistic effect of the two drugs against this pathogen. Our previous study also demonstrated the synergistic effect of combining baicalin with tobramycin against CRPA. The biofilm formation ability and biofilm activity of CRPA decreased under the combined effect of baicalin and tobramycin in a non-dose-dependent manner. In this study, RT-polymerase chain reaction (PCR) was used to investigate the synergistic effect of combining baicalin with tobramycin on the expression of carbapenem resistance genes *VIM*, *IMP* and *OprD2* and biofilm-related genes *algD*, *pslA* and *lasR* in CRPA. In addition, tobramycin combined with baicalin medication group was compared with tobramycin alone medication group to study the effect of baicalin combined with tobramycin on CRPA.

MATERIALS AND METHODS

Bacteria and medicines

A total of 30 tobramycin-susceptible CRPA strains were collected from inpatients in our hospital from 2018 to 2020. Repeated strains isolated from the same part of the same patient were excluded from this count. All the 30 strains screened met the "Regulations on the Administration of Medical Institutions" of the State Council. The quality control strain ATCC27853 was provided by Mérieux (France). Both baicalin and tobramycin were purchased from Shanghai Maclean Biochemical Technology Co., Ltd (China).

Reagents and equipment

The following reagents were used: Columbia blood agar plate (Mérieux, France), lysostaphin (Shanghai Yuanye Biotechnology Co., Ltd., China), quantitative PCR reagent SG Fast qPCR Master Mix (ABI) and absolute ethanol (Jiangxi Grass Coral Disinfection Products Co., Ltd.). The following equipment were used: Ezup column bacterial genomic DNA extraction kit (Shanghai Sangon Bioengineering Co., Ltd.), 96-well cell culture plate (NEST company), common bacteria incubator (Shanghai Boxun Biotechnology Co., Ltd., China), VITEK turbidimeter (BioMérieux China Co., Ltd., China), Micropipette (Xingchuang Experimental Instrument Co., Ltd., China), Micro Vortex Mixer (Shanghai Huxi Analytical Instrument Factory Co., Ltd., China), Electrothermal Thermostat (Shanghai Yuejin Medical Instrument Co., Ltd., China), Small low-speed centrifuge (Thermo company), SW-CJ-1D clean workbench (Jiangsu Sujie equipment factory), PCR reaction amplifier (BIO company), StepOne fluorescence quantitative PCR instrument (ABI) and Stepone plus fluorescence Quantitative PCR instrument (ABI).

CRPA DNA extraction

Briefly, the reserved experimental strains were inoculated on Columbia blood plates and cultured overnight at 35°C. After 18–24 h, a single pure colony was inoculated into 4 mL sterile LB broth with shaking at 35°C for 18–24 h. The concentration was then adjusted to 0.5 McDonnell's with sterile physiological saline. A certain amount of bacterial solution was diluted 100 times with sterile solution. To prepare bacterial culture, bacteria containing a certain concentration of tobramycin, baicalin, and tobramycin solution (1, 1/2, 1/4, 1/8 MIC) in LB broth (equivalent to 5×10^5 cfu/mL) was incubated at 35°C for 24 h. For DNA extraction, 1 mL of this bacterial culture was added to a 1.5 mL centrifuge tube and centrifuged at 8000 rpm for 1 min at room temperature, with the supernatant discarded and the precipitate retained as DNA.

Tobramycin resistance gene and biofilm gene detection

PCR and RT-PCR were used to detect the expression of CRPA genes resistant to tobramycin, baicalin, and tobramycin combined with baicalin and biofilm-related genes, including carbapenem resistance genes *VIM*, *IMP* and *OprD2*, and biofilm-related genes *algD*, *pslA*, and *lasR*. With 16SrRNA as the reference gene, the differences in the expression profile of drug resistance genes in different groups were analyzed in comparison with the blank control group. The primers were adapted from previous publications. The primer sequences are shown in Table 1.

From 2016 to 2020, patients admitted to our hospital with CRPA bloodstream infection were included. Their clinical and microbiological data were comprehensively collected. The inclusion criteria were as follows: (1) Inpatients aged ≥ 18 years; (2) having complete clinical data; and (3) ≥ 1 blood culture specimen of *P. aeruginosa* with positive culture and clinical evidence of corresponding bloodstream infection and multiple cultures of *P. aeruginosa* strains collected from the same patient. This study analyzed the first cultured strains.

Patients were divided into death group and survival group according to whether the patients died during hospitalization or not. Univariate analysis was performed on the clinical characteristics, laboratory indicators and treatments of the two groups of patients. Subsequently, univariate analysis results with $P < 0.05$ were included in the multivariate analysis to explore independent risk factors for patient mortality.

Statistical analysis

The SPSS version 26.0 statistical software was used to analyze and process the results. Chi-square test was used to analyze count data, and *t*-test was used for comparison between two samples. Logistic regression analysis was used for multivariate analysis, and the odds ratio and 95%CI were calculated. Values with $P < 0.05$ were considered statistically significant.

RESULTS

Determination of the relative expression levels of CRPA resistance genes and biofilm-related genes using RT-PCR

The relative expression levels of CRPA resistance genes and biofilm-related genes varied under different drug concentrations, as shown in Table 2.

As shown in the Table 2, the drug resistance genes *VIM* and *IMP* were significantly down-regulated in both the single-use group and the combination group. Similarly, most of the biofilm-related genes, including *algD*, *pslA* and *lasR* were significantly down-regulated. In contrast, the *OprD2* combination and baicalin and tobramycin alone were up-regulated. *Mycin* was down-regulated, whereas *lasR* was slightly up-regulated in the baicalin-alone group.

Table 1 Polymerase chain reaction primers used

Gene	Sequence	Tm (°C)
VIM-1	RTF:GTTTG GTCGC ATATC GCAAC	60
VIM-2	RTR:AATGC GCAGC ACCAG GATAG	60
IMP-1	RTF:GAAGG YGTTT ATGTT CATAAC	60
IMP-2	RTR:GTAMG TTTCA AGAGT GATGCC	60
OprD-1	RTF:ATGAA AGTGA TGAAG TGGAG CG	60
OprD-2	RTR:TTACA GGATC GACAG CGGAT AG	60
algD-1	RTF:CGAGAAAGTCCGAACGCCACAC	60
algD-2	RTR:ATCGGCGGGAAGTCGTA	60
pslA-1	RTF:GGCCTGTTCCCTACCT	60
pslA-2	RTR:GCGGATGTCGTGGTTG	60
lasR-1	RTF:GAAGATGGCGAGCGACCTTGGATTC	60
lasR-2	RTR:CTCGTGCTGCTTTCGCGTCTGGTAG	60
16sRNA	RTF:ACTCCTACGGGAGGCAGCAG	60
16sRNA	RTR:ATTACCGCGGCTGCTGG	60

Correlation between CRPA biofilm formation and related gene expression

Table 3 shows the Pearson correlation between the amount of biofilm formation and the expression of related genes.

The experimental data revealed a certain correlation between the biofilm formation of the strain and the expression of related genes (Table 3). Significant correlations were found between biofilm volume and VIM (PCC = 0.862, $P < 0.01$), IMP and VIM (PCC = 0.761, $P < 0.05$), OprD2 and IMP (PCC = 0.811, $P < 0.05$), pslA and OprD2 (PCC = 0.683, $P < 0.01$) and lasR and pslA (PCC = 0.935, $P < 0.01$).

Patient clinical data

After applying the inclusion criteria, a total of 57 patients were enrolled in this study, including 22 in the tobramycin combined with baicalin group and 35 in the tobramycin-only group, with a mean age of 58 years. The baseline data for all patients with CRPA bloodstream infections are shown in Table 4. Among the 57 patients, 36 were admitted to the intensive care unit, and 12 were mechanically ventilated. During follow-up, 17 patients died and the remaining patients survived.

Therapeutic effect of baicalin combined with tobramycin

This paper analyzed clinical variables associated with patient outcomes. The results of univariate analysis showed that APACHE II score, albumin, combined surgical treatment and baicalin combined with tobramycin were significantly associated with the prognosis of patients. As can be seen from the multivariate analysis results shown in Table 5, patients receiving baicalin in combination with tobramycin had a significant improvement in prognosis. Table 5 presents the multivariate logistic regression analysis of death group and survival group in *P. aeruginosa* bloodstream infection.

DISCUSSION

P. aeruginosa is a non-fermenting gram-negative bacillus that is a common opportunistic pathogen[9]. It is highly adaptable to external environments and can survive in a humid environment for a long time [10]. When *P. aeruginosa* contaminates medical water or medical equipment, it easily forms biofilms that are difficult to remove, which often leads to hospital-acquired infections[11]. In recent years, with the widespread use of antibiotics, *P. aeruginosa* has acquired resistance genes, developing multidrug resistance, pan-drug resistance and even complete resistance[5]. CRPA is resistant strain of *P. aeruginosa* that poses a great challenge to current clinical treatment[12].

Studies have shown that the current CRPA resistance mechanisms mainly include carbapenemase production, outer membrane permeability protein deletion, and active efflux pump mechanisms[13]. Metallo- β -lactamases (MBLs) are a group of carbapenemases that can extensively hydrolyze β -lactam antibiotics[14]. At present, the MBLs produced by *P. aeruginosa* are detected in all clinical settings, including the VIM and IMP families, especially VIM-2[15]. In contrast, the domestic *P. aeruginosa*

Table 2 Differences in relative expression levels between related genes

Index	Group	Assay name	ΔCt	$\Delta\Delta Ct$	Fold change	Up/down
1	Baicalin	VIM	15.94	2.63	0.16	Down
	Tobramycin	VIM	16.81	3.50	0.09	Down
	Baicalin + tobramycin	VIM	18.09	4.78	0.04	Down
	Control	VIM	13.31		1.00	
2	Baicalin	IMP	13.73	2.58	0.17	Down
	Tobramycin	IMP	13.49	2.34	0.20	Down
	baicalin+ tobramycin	IMP	13.56	2.41	0.19	Down
	Control	IMP	11.15		1.00	
3	Baicalin	OprD2	10.13	-0.11	1.08	Up
	Tobramycin	OprD2	11.40	1.16	0.45	Down
	Baicalin + tobramycin	OprD2	9.64	-0.60	1.52	Up
	Control	OprD2	10.24		1.00	
4	Baicalin	algD	1.88	0.37	0.77	Down
	Tobramycin	algD	2.23	0.72	0.61	Down
	Baicalin + tobramycin	algD	3.44	1.93	0.26	Down
	Control	algD	1.51		1.00	
5	Baicalin	pslA	3.29	0.63	0.65	Down
	Tobramycin	pslA	3.11	0.45	0.73	Down
	Baicalin + tobramycin	pslA	3.17	0.51	0.70	Down
	Control	pslA	2.66		1.00	
6	Baicalin	lasR	1.18	-0.09	1.06	Up
	Tobramycin	lasR	1.43	0.16	0.90	Down
	Baicalin + tobramycin	lasR	1.66	0.39	0.76	Down
	Control	lasR	1.27			

Table 3 Pearson correlation between the amount of biofilm formation and the expression of related genes

	RB	VIM	IMP	OprD2	algD	pslA	lasR
RB	1	0.862	0.373	-0.3548	0.178	0.223	-0.001
VIM		1	0.761	0.287	0.319	0.1321	0.007
IMP			1	-0.811		0.0505	0.471
OprD2				1	0.3155	0.683	0.214
algD					1	0.4772	0.691
pslA						1	0.935
lasR							1

Significant correlations were found between biofilm volume and VIM ($P < 0.01$), IMP and VIM ($P < 0.05$), OprD2 and IMP ($P < 0.05$), pslA and OprD2 ($P < 0.01$) and lasR and pslA ($P < 0.01$).

detections are mainly VIM, IMP and SPM types[16]. Channel proteins are embedded in the lipid bilayer of *P. aeruginosa*, which is a non-specific, water-soluble diffusion channel that spans the cell membrane. Among them, the outer membrane proteins OprC, OprD2 and OprE show strong pore activity[17]. Scofield *et al*[18] and Wang *et al*[19] reported that OprD2 gene deletion is the main mechanism of *P. aeruginosa* resistance to imipenem. Quorum sensing is a general mechanism of bacterial cell-to-cell

Table 4 Baseline characteristics of patients with carbapenem-resistant *Pseudomonas aeruginosa* bloodstream infection

Clinical variables	Patients (n = 57)
Age (yr)	58 ± 23
Length of hospital stay before infection (d)	24 ± 5
Combeities	
Blood system disease	12
Solid tumor	11
Admission to intensive care unit	36
APACHE II	19 ± 7
TTP	18 ± 6
Albumin < 30 g/L	23
Underwent surgery	18
Invasive mechanical ventilation	12
Antibiotic application ≥ 7 d	37
Outcome	
Sepsis and shock	22
Death	17

Table 5 Multivariate logistic regression analysis of death group and survival group in *Pseudomonas aeruginosa* bloodstream infection

Clinical variables	Odds ratio	95%CI	P value
APACHE II	1.27	1.02-1.58	0.033
Albumin < 30 g/L	6.72	1.18-32.57	0.035
Underwent surgery	3.56	1.03-6.22	0.048
The baicalin in combination with tobramycin	0.56	0.42-0.73	0.027

information transfer, which controls the behavior of entire bacterial populations by synthesizing and secreting signaling molecules (also known as autoinducing molecules)[20]. When signal molecules reaches a certain concentration threshold in bacterial population density, the expression of some specific genes is activated, which regulates the adaptive function of bacterial populations. *P. aeruginosa* strains are characterized by quorum sensing systems, mainly Las quorum sensing systems[21].

The Las system consists of *LasR* and *LasI* genes. The Las system is associated with *P. aeruginosa* infection and can increase its resistance to certain antibiotics, such as imipenem and ciprofloxacin[22]. The *algD* gene is the first gene encoding an alginate biosynthesis enzyme operon. The transcriptional activation of this gene is associated with the synthesis of alginic acid[23]. It has been reported that acid salts play an important role in the formation of *P. aeruginosa* biofilms[24]. As a highly conserved sequence, *pslA* is the first gene encoding glucotransferase on the *P. aeruginosa* *psl* operon. It has been reported in the literature that both of these two-polysaccharide synthesis-related genes play key roles in *P. aeruginosa* adhesion and biofilm formation[25]. Therefore, in this study, the biofilm-related genes *LasR*, *algD* and *pslA* and drug resistance genes *VIM*, *IMP* and *OprD2* were detected in different groups of gene changes, and the association between these genes and the synergistic effect of the combination drug were analyzed.

This experiment showed that baicalin, tobramycin and baicalin combined with tobramycin group had different effects on gene expression. Pearson's correlation analysis results revealed a correlation between the amount of biofilm formation and the expression of *VIM*, *IMP*, *OprD2*, *algD*, *pslA* and *lasR* genes. The most significant correlations were as follows: the amount of biofilm and *VIM*, *OprD2* and *IMP*, *pslA* and *OprD2*, *lasR* and *pslA*. The drug resistance genes *VIM* and *IMP* were significantly down-regulated in the single-use group and the combined drug group. Similarly, most of the biofilm-related genes *algD*, *pslA*, and *lasR* were significantly down-regulated, indicating that the three groups of drugs were all effective against CRPA, with better efficacy in combination than as single medication. The up-regulation of *OprD2* combined with baicalin alone was consistent with the results of Scofield *et al*[18]. However, the down-regulation of tobramycin alone and the slight up-regulation of *lasR* in the baicalin group was different, probably due to individual differences among strains, including their inhibition ratios. Other

drug resistance mechanisms may also account for this difference. Therefore, the mechanism of action of *P. aeruginosa* is complex and requires further research.

CONCLUSION

The fatality rate among patients with *P. aeruginosa* bloodstream infection is high[26]. Many factors account for the poor prognosis of patients. These can be divided into host including advanced age, APACHE II score, underlying diseases such as hematological diseases and malignant tumors, and septic shock, and iatrogenic factors such as irrational initial antibiotic treatment[27]. The multivariate logistic regression analysis of death in this study showed that APACHE II score, combined surgery, and albumin < 30 g/L were independent risk factors for death[28]. A foreign study involving 187 cases of hospital-acquired *P. aeruginosa* bloodstream infection showed that the combined use of empiric antibiotics could reduce the mortality of infected patients[29]. Further multivariate analysis showed that empiric sensitive antibiotic therapy could reduce the risk of infection. For patients with suspected infection, sensitive drugs should be empirically used early. Our results showed that patients treated with baicalin combined with tobramycin had significantly better prognosis compared with those treated with tobramycin alone. Therefore, baicalin combined with tobramycin therapy shows potential as an effective treatment for patients with CRPA infection.

ARTICLE HIGHLIGHTS

Research background

Pseudomonas aeruginosa (*P. aeruginosa*) is an important cause of nosocomial infections, and contributes to high morbidity and mortality, especially in intensive care units. *P. aeruginosa* is considered a 'critical' category bacterial pathogen by the World Health Organization to encourage an urgent need for research and development of new antibiotics against its infections.

Research motivation

In this study, the anti-bacterial effect of baicalin combined with tobramycin on carbapenem-resistant *P. aeruginosa* (CRPA) infection was investigated, including the potential synergistic effect of the two drugs against this pathogen.

Research objectives

The objective of this research is to analyze the distribution of CRPA in a specific hospital over a period of time, and to investigate the effectiveness of baicalin combined with tobramycin therapy as a potential treatment method for CRPA infections. The study aims to explore the correlation between biofilm formation and the expression of drug-resistant and biofilm-related genes in CRPA, with the goal of identifying potential targets for effective treatment.

Research methods

The expression levels of drug-resistant genes and biofilm-related genes in CRPA that confer resistance to tobramycin, baicalin and tobramycin combined with baicalin were detected.

Research results

There was a correlation between biofilm formation and the expression of biofilm-related genes. In addition, *VIM*, *IMP*, *OprD2*, *algD*, *pslA* and *lasR* that confer biofilm production under different concentrations in CRPA were significantly correlated. The synergistic effect of baicalin combined with tobramycin was a significant down-regulation of *VIM*, *IMP*, *algD*, *pslA* and *lasR*.

Research conclusions

Baicalin combined with tobramycin therapy can be an effective treatment method for patients with CRPA infection.

Research perspectives

Future research can focus on further investigating the effectiveness of baicalin combined with tobramycin therapy in the treatment of CRPA infections, including exploring optimal dosage and administration methods. Additionally, further studies can explore the mechanisms underlying the down-regulation of drug-resistant and biofilm-related genes by baicalin and tobramycin, as well as potential side effects or limitations of this treatment method. Moreover, more research could be conducted to investigate other potential treatments for CRPA infections and to evaluate their clinical efficacy. Finally, efforts can be made to develop new approaches for preventing and controlling the

spread of CRPA in hospitals and healthcare settings.

FOOTNOTES

Author contributions: Jin LM is mainly responsible for experimental design and writing articles; Shen H is mainly responsible for collecting data and samples; Chen XY conducted experimental operations and conducted statistical analysis of articles; Jin Y and Yuan CM are responsible for specimen selection and experimental operations; Zhang NH is responsible for experimental guidance and important revisions to the article, and all authors read and approved the final version.

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