Life Science Informatics Publications
Research Journal of Life Sciences, Bioinformatics, Pharmaceutical and Chemical Sciences
Journal Home page http://www.rjlbpcs.com/
RJLBPCS
ISSN 2454-6348

Original Research Article **DOI: 10.26479/2018.0403.40**

ANTIBACTERIAL ACTIVITY OF A COMBINATION OF CYSTEINE AND CIPROFLOXACIN AND ITS RELATIONSHIP WITH THE GENERATION OF OXIDATIVE STRESS IN EXTENDED-SPECTRUM BETA-LACTAMASE-PRODUCING *ESCHERICHIA COLI* STRAINS

SR Martínez [1,2], ME Bongiovanni[3], A Piersigilli[4], I Albesa[2], MC Becerra[1,2*]

1. Instituto Multidisciplinario de Biologia Vegetal (IMBIV), CONICET, Facultad de Ciencias Químicas, Universidad Nacional de Cordoba, X5000HUA, Cordoba, Argentina.

2.Departamento de Ciencias Farmaceuticas, Facultad de Ciencias Químicas, Universidad Nacional de Cordoba, X5000HUA, Cordoba, Argentina.

3. Hospital Privado. Av. Naciones Unidas 346, B° Parque Velez Sarsfield CPA X5016KEH, Cordoba, Argentina.

4. Sanatorio Privado Aconcagua, Rondeau 455 X5000IIR, Cordoba, Argentina.

ABSTRACT: The aim of the present work was to evaluate whether L-cysteine enhances the antibiotic susceptibility of extended-spectrum beta-lactamase (ESBL) *Escherichia coli* strains to ciprofloxacin and to identify the role of Reactive oxygen species (ROS) in the antimicrobial activity. The combined ef5fect of L-cysteine and ciprofloxacin was investigated by the macrodilution broth method and chemiluminescence assay. ESBL 1 and ESBL 2 strains presented a growth inhibition when they were incubated with 0.4 or 0.2 mM of cysteine, respectively compared to the control without antibiotic. Both strains at sub-inhibitory concentration of ciprofloxacin were combined with cysteine, and a clear growth inhibition respect to the control was observed. An increase of 68% in ROS occurred respect to the control when ESBL 1 was treated with a combination of ciprofloxacin and cysteine, while for ESBL 2 there was a rice of 127 %. The combination of ciprofloxacin with cysteine had the capacity to undergo redox cycling and ROS production with subsequent cellular injury.

KEYWORDS: antibiotic; L-cysteine; radicals; *Escherichia coli*.

Corresponding Author: Dr. MC Becerra* Ph.D.

1. Instituto Multidisciplinario de Biología Vegetal (IMBIV), CONICET, Facultad de Ciencias Químicas, Universidad Nacional de Cordoba, X5000HUA, Cordoba, Argentina.

2.Departamento de Ciencias Farmacéuticas, Facultad de Ciencias Químicas, Universidad Nacional de Cordoba, X5000HUA, Cordoba, Argentina.

Email Address: becerra@fcq.unc.edu.ar

1.INTRODUCTION

The emergence of *Escherichia coli* being resistant to antibiotics is a problem that affects various countries. Organisms expressing extended-spectrum beta-lactamases (ESBL) are widely distributed worldwide, although prevalence rates are significantly higher in certain geographical regions [1]. ESBLs are in the 2be group of the Karen Bush classification derived from specific mutations of genes encoding betalactamases e.g. TEM-1, TEM-2 and SHV-1. These confer resistance to all beta-lactam antibiotics except cephamycins and carbapenems and may also confer resistance to other classes of antibiotics; such as aminoglycosides, quinolones, trimethoprim, sulphonamides, tetracyclines and chloramphenicol, due to the plasmids carrying the genes coding for ESBLs. In addition, ESBL generally contain other genes of resistance to various antibiotics, thus delimiting further the therapeutic options available against infections caused by enterobacteria with ESBL [2-4]. Quinolone resistance and ESBL production are often associated in *Enterobacteriaceae* and are cross-resistant to quinolones and beta-lactams. Hence, the prescription of one of these categories of antibiotics may consequently be selected for bacteria which are resistant to both categories [5]. The generation of reactive oxygen species (ROS) is an inevitable aspect of life under aerobic conditions. These are continuously produced as side-products of certain metabolic pathways and by some specific systems, with oxidative stress investigation related to different applied aspects including medicine having become increasingly popular [6]. Our previous results indicated that the antibiotics used in clinical treatments such as fluoroquinolones, chloramphenicol, ceftazidime, among others, induce oxidative stress in different bacterial species [7-10]. In this sense, we have demonstrated that ciprofloxacin increases superoxide anion (O_2^-) in sensitive strains of *Staphylococcus aureus*, which is accompanied by an evident rise in 8-oxodG, considered to be the major DNA marker of oxidative stress [11]. In addition, it was previously reported that bacterial gyrase inhibitors such as synthetic quinolone antibiotics promoted the formation of hydroxyl radicals which contributed to cell death [12]. Several years ago, it was described that cysteine is bactericidal to or inhibits the growth of a variety of microorganisms, including, *Salmonella typhimurium*. In addition, this amino acid undergoes a metal ion-catalyzed autoxidation, leading to hydrogen peroxide production [13].

Cysteine has also been shown to inhibit RNA synthesis as well as branched-chain amino acid synthesis in *E. coli,* and it has been suggested that these metabolic alterations may result in an unbalanced growth of this organism. In *E. coli*, cysteine toxicity has been shown to be due to a cysteine-specific inhibition of the branched-chain amino acid synthesis [14]. Taking this background into account, our aim of the present study was to evaluate whether cysteine enhances the antibiotic susceptibility of *E. coli* ESBL strains to ciprofloxacin, and to identify more precisely the role of ROS in the antimicrobial activity.

2. MATERIALS AND METHODS

Drugs

Chemicals. The L-cysteine and bis-N-methylacridinium nitrate (lucigenin) used were products of Sigma Chemical Co., St. Louis, Mo. The antibiotic ciprofloxacin HCl was purchased from Parafarm.

Bacterial strains and antibiotic susceptibility test.

Two extended-spectrum beta-lactamase-producing *E. coli* (ESBL) strains (ESBL 1 and ESBL 2) from hospitalized patients were kindly provided by Sanatorio Aconcagua from Córdoba, Argentina. Stock cultures were maintained in tryptic soy broth and stored frozen in 10% of glycerol. The screening and confirmatory methods to detect the presence of ESBLs were used according to the Clinical and Laboratory Standard Institute (CLSI: M100-S20, 2015) [15].

Evaluation of cysteine toxicity in *Escherichia coli* ESBL strains.

To evaluate the potential toxicity of cysteine toward two strains of *E. coli* (ESBL strain 1 and ESBL strain 2), minimal medium M-9 was used. This medium was prepared with the following formula per liter of distilled water: 6.8 g Na_2HPO_4, 3.0 g of KH_2PO_4, 1.0 g of NH_4Cl, 0.5 g NaCl; final pH 6.8 ± 0.2 at 25 ° C, and the mixture was sterilized by the method of pressurized steam at 121 °C for 15 minutes. Aseptically, 20 mL of sterile of 20% glucose and 2 mL of sterile 1 M magnesium sulfate were added [13]. Both *E. coli* suspensions with a final concentration of approximately $1x10^6$ CFU/mL (colonies forming units per mL) were prepared from overnight cultures in M-9 medium. Each suspension was exposed to different concentrations of cysteine (ranging from 0.05 to 1.6 mM) by adding an appropriate volume of cysteine stock solution to reach a final volume of 1 mL, which was incubated at 37 ºC for 24 h. A control inoculum of each strain (culture prepared in minimal medium without cysteine) was also run in parallel. After being incubated overnight at 37°C, the turbidity was observed.

MIC determination

The MICs of ESBL 1 and ESBL 2 of ciprofloxacin were determined in a minimal medium M-9 by using the tube dilution method. Suspensions of *E. coli* strains (ESBL 1 and ESBL 2) were prepared from overnight cultures and diluted to obtain a final concentration of approximately $5x10^5$ CFU/mL, after which ciprofloxacin in the range of 0.083 to 1.33 mM was added. Bacterial growth was

observed at 24 h of incubation at 37 °C.

Checkerboard Assay

The interaction between cysteine and ciprofloxacin was evaluated by the checkerboard method [16]. Bacterial growth inhibition resulting from the interactions was determined by the macrodilution test. The concentrations of each tested agent used in the combinations corresponded to serial 2-fold dilutions. The concentrations of cysteine were ranged from 0.05 to 0.8 mM in medium M-9. Dilutions of ciprofloxacin (0.083 to 1.33 mM) were also prepared in the same manner. An overnight culture of each microorganism was diluted to achieve a cell density in the range of $5x10^5$ CFU/mL. The fractional inhibitory concentration (FIC) was calculated using the MIC from the checkerboard assay and the MIC of each compound alone, obtained in parallel in the same assay, according to the following formula: FIC=MIC of antimicrobial agent in combination/MIC of antimicrobial agent alone. Then, the synergistic effect was evaluated by calculating the FICindex (FICI) for each combination, by adding the individual FIC values. A FICI of 0.5 indicated synergy for the combination. When it fell between 0.5 and 1, it was defined as an additive effect, and between 1.0 and 4.0 it was classified as "no interaction". Finally, a FICI >4.0 indicated antagonism between the components in the combination.

Determination of ROS by the chemiluminescence (CL) assay.

Oxidative stress was investigated in a BioOrbit luminometer and the chemiluminescence assay was quantified by the light emitted when ROS gave electrons to bis-N-methylacridinium nitrate (lucigenin, Sigma). Suspensions of *E. coli* strains (ESBL 1 and ESBL 2) were prepared from overnight cultures in a minimal medium (M-9) and optical densities (OD) were measured at 600 nm and diluted to obtain an OD of 0.05. Then, 50 µL of these suspensions were added to 5 mL of M-9 medium and incubated for 2 h at 37°C to reach the log-phase growth. Then, 0.1 mL of these suspensions were incubated with 0.1 mL of lucigenin (75 µg/mL) and 0.1 mL of phosphate buffer saline (PBS, pH 7.4), and immediately following this, 0.1 mL of ciprofloxacin (at concentrations corresponding to the MIC), 0.1 mL of cysteine (0.8 mM) or the association of both compounds was added. Finally, the reaction was triggered with 0.1 mL of dimethylsulfoxide (DMSO). Basal controls of ROS production were made with bacterial suspension in the absence of antibiotic or cysteine. The light emitted by ROS was expressed as relative light units (RLU) at various times in seconds, with the subtraction of the background.

Statistical methods

Statistical analysis was assessed by the Student's *t*-test and $p < 0.05$ was taken as being statistically significant. The experiments were repeated at least three times.

3. RESULTS AND DISCUSSION

Evaluation of the association of cysteine with ciprofloxacin in *Escherichia coli* strains

The MIC of ciprofloxacin obtained for ESBL 1 was 0.33 mM and 0.165 mM for ESBL 2 in M-9 medium. Cultures of strains ESBL 1 and ESBL 2 presented a growth inhibition when they were incubated with 0.4 mM and 0.2 mM cysteine compared to the control, respectively.

Synergy studies

Cysteine plus ciprofloxacin combination exhibited a synergistic effect for the strain ESBL 1 (Table 1, inset A). A FIC of 0.125 was obtained with ciprofloxacin concentration of 0.66 mM, when

Table 1. Minimum Inhibitory Concentrations (MICs) and Fractional Inhibitory Concentration Indices (FICIs) of cysteine in combination with ciprofloxacin against *Escherichia coli* ESBL 1 and ESBL 2.

A.

ESBL 1	ciprofloxacin (MIC = 0.66 mM)		
cysteine (MIC = 0.4 mM)			
$MIC_{cys-cip}$	**FIC**	**$MIC_{cip-cys}$ = 0.083 mM**	**FIC = 0.125**
0.1	0.25	$FICI_{cys-cip}$= 0.375	
0.15	0.375	$FICI_{cys-cip}$= 0.5	
0.2	0.5	$FICI_{cys-cip}$= 0.625	

B.

ESBL 2	ciprofloxacin (MIC = 0.33 mM)		
cysteine (MIC = 0.2 mM)			
$MIC_{cys-cip}$	**FIC**	**$MIC_{cip-cys}$ = 0.083 mM**	**FIC = 0.25**
0.05	0.25	$FICI_{cys-cip}$= 0.5	
0.1	0.5	$FICI_{cys-cip}$= 0.75	
0.15	0.75	$FICI_{cys-cip}$= 1	

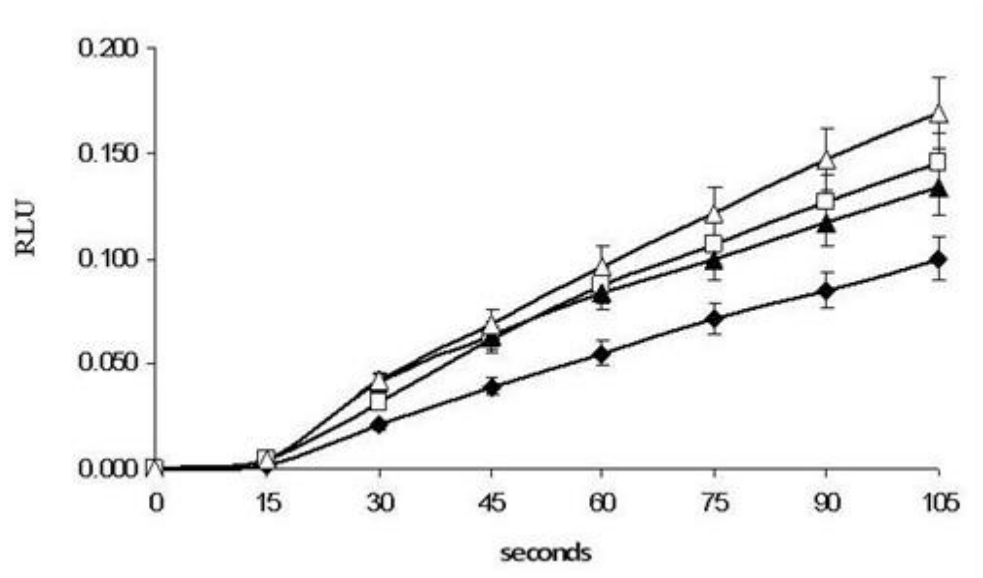
$MIC_{cys-cip}$: MIC of cysteine in combination with ciprofloxacin. $MIC_{cip-cys}$: MIC of ciprofloxacin in combination with cysteine. FIC: fractional inhibitory concentration. $FICI_{cys-cip}$: fractional inhibitory concentration index (FIC of cysteine plus FIC of ciprofloxacin).

cysteine was combined with this antibiotic, a value of $FICI_{cys-cip}$ of 0.375 was achieved. In the case of ESBL2, a FIC of 0.25 was gained at 0.33 when the concentration of ciprofloxacin was 0.33 mM, when combined with cysteine, a value of $FICI_{cys-cip}$ of 0.5 was accomplished (Table 1, inset B).

Determination of ROS by the chemiluminescence (CL) assay.

Chemiluminescence assays revealed a higher degree of oxidative stress in the ESBL 2 strain compared to ESBL 1. When this latter strain was treated with a combination of ciprofloxacin at 1.39 mM and 0.8 mM of cysteine, the maximum value of RLU reached was 0.183 ± 0.030, representing an increase of 68% respect to the control without antibiotic or cysteine (Figure 1A).

A



B

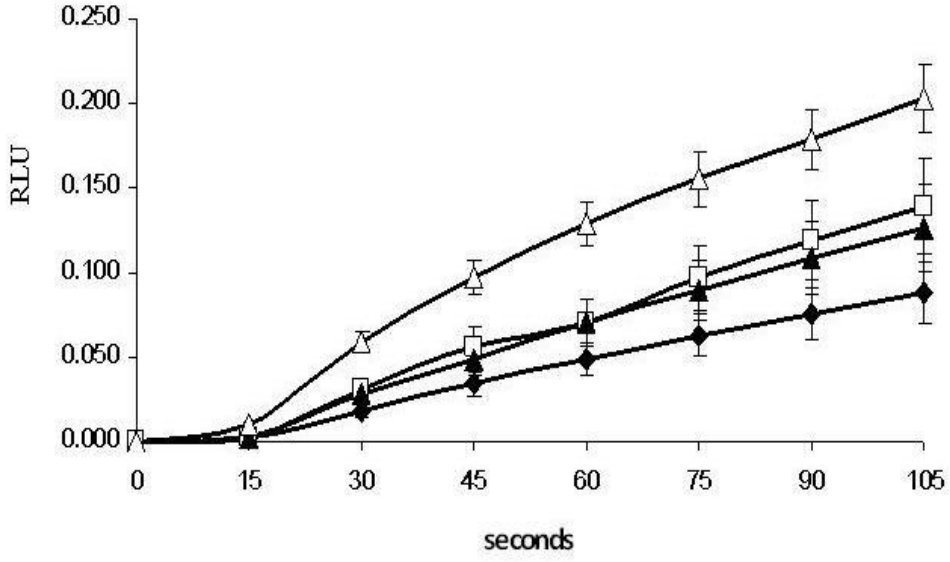


Figure 1. ROS generation in *E. coli* determined by chemiluminescence assay. *A*. ESBL 1 strain. Control (♦), treated with 1.39 mM ciprofloxacin (□), 0.8 mM cysteine (▲), or a combination of ciprofloxacin and cysteine (Δ). *B*. ESBL 2 strain. Control (♦), treated with 0.69 mM ciprofloxacin (□), 0.8 mM cysteine (▲), or a combination of ciprofloxacin and cysteine (Δ).

While for the ESBL 2 strain, the maximum value of RLU attained was 0.216 ± 0.059, which was an increase of 127 % respect to the control (Figure 1B). These results agreed with the level of susceptibility of each strain to the antibiotic, since ESBL 1 was more resistant than ESBL 2 and this could have been related to less generation of ROS occurring in this strain. The first reports on ESBLs in Argentina date back to 1990. Since then, dispersion has been observed between different *Enterobacteria* and other Gram-negative bacilli [17,18] which create a problem for therapy. We have previously demonstrated that *E. coli* can react efficiently in the presence of substances that alter the level of ROS and have also applied chemiluminescence in the study of the oxidative alterations generated in bacteria by antibiotics, including ceftazidime and ciprofloxacin [7, 19]. The relationship between antibiotic action and oxidation in clinical strains was also demonstrated in *Proteus mirabilis* treated with ciprofloxacin by Aiassa *et al.* [9]. Thus, it was interesting to investigate the ability of a combination of ciprofloxacin with cysteine to undergo oxidative stress. In this work, we have evaluated the effect of ciprofloxacin against Gram negative bacteria. Ciprofloxacin targets the DNA topoisomerases and DNA gyrase [20-21]. The studied strains were found to be resistant to the antibiotic. However, association with cysteine increased the susceptibility and inhibited bacterial growth. The results obtained by chemiluminescence assays confirmed that the growth inhibition of ESBL 1 and ESBL 2 strains exposed to the antibiotic and L-cysteine was associated with ROS generation. In cytotoxicity assays, Kartal-Hodzic et al. showed that L-cysteine was not toxic to the Caco-2 cells until a concentration of 9.9 mM was reached. The results of this toxicity study are very interesting because it demonstrated that L-cysteine might be able to be used in different formulations [22]. Previously, was reported that L-cysteine and L-cystine greatly potentiated the bactericidal effect of hydrogen peroxide in *E. coli* K-12 [23]. Although a bacteriostatic or bactericidal effect of cysteine on various microorganisms has been known for a long time, the present study is the first report which demonstrates that L-cysteine potentiates the bactericidal effect of ciprofloxacin, even in bacteria with important resistance to antibiotics. Furthermore, these findings support our hypothesis that targeting the respiratory chain could be a strategy to increase the susceptibility to antibiotics of resistant strains.

4. CONCLUSION

Summing up, the combination of ciprofloxacin and cysteine has the capacity to undergo redox cycling and ROS production with subsequent cellular injury. The results of this work reveal new perspectives for increasing the antibiotic effects related to the generation of oxidative stress in extended-spectrum beta-lactamase-producing *Escherichia coli* strains.

5.ACKNOWLEDGEMENT

The authors gratefully acknowledge the financial support granted by Secretaría de Ciencia y Tecnología from the Universidad Nacional de Córdoba (SECyT-UNC), Consejo Nacional de

Investigaciones Científicas y Tecnológicas (CONICET) and Ministerio de Ciencia y Tecnología (MINCyT). M.C.B is a career research member of CONICET and SRM a postdoctoral fellow of CONICET.

6. CONFLICT OF INTEREST

The authors declare no conflict of interest

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