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RELATIONSHIP BETWEEN OXIDATIVE AND NITROSATIVE STRESS INDUCED BY GENTAMICIN AND CIPROFLOXACIN IN BACTERIA

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AUTHORS' CONTRIBUTIONS

This work was carried out in collaboration between all authors. Authors PLP and ILDG designed the study, wrote the protocol and interpreted the data. Author ILDG anchored the field study, gathered the initial data and performed preliminary data analysis. Authors PLP and PMG managed the literature searches and produced the initial draft. All authors read and approved the final manuscript.

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ABSTRACT

Oxygen is essential to the life of the aerobic organisms, and most is used for the intracellular generation of energy, where several molecules are formed by partial reduction of oxygen. Because of its short half-life, low selectivity and high reactivity, biological targets of reactive oxygen species (ROS) include various components such as proteins, nucleic acids and lipids. The purpose of this work was to establish a relationship between ROS and reactive nitrogen intermediates (RNI) generation in bacteria exposed to antibiotics. ROS was determined by the reduction of nitro blue tetrazolium (NBT). In aqueous solutions, nitric oxide is converted to nitrite (NO_2^-), this was determined using a non-enzymatic colorimetric assay. NBT studies indicated stimulation of oxidative stress with an increase of ROS generated by the antibiotic in the bacteria. We observed an increase in the production of NO_2^- dose-dependent with CIP and GEN. The determination of ROS and RNI allowed establish differences in the alteration of oxidative metabolism in susceptible and resistant strains. Resistance to CIP and GEN may be related to the decreased production of ROS and RNI and consequently, a smaller macromolecules bacterial oxidation. These results contribute to a better understanding of the ROS and RNI regulation.

Keywords: Oxidative stress; bacteria; nitrosative stress; antibiotics.

1. INTRODUCTION

Oxidative stress is caused by cell exposure to reactive oxygen species (ROS) such as superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO^{\bullet}$), which can damage proteins, nucleic acids and cell membranes. As a consequence, generates a variety of biochemical and physiological changes that can lead to various forms of cellular damage. Recent studies suggest that the effects of these oxidants are linked to damage caused by reactive nitrogen species (RNS) such as nitric oxide (NO), peroxynitrite ($OONO^-$) and nitrosothiols (RSNO). To counteract oxidative stress censing cells increases ROS and transduce signals to increase their defenses [1,2].

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Reactive oxygen species can also be generated by external factors such as exposure to radiation, light, metals, antibiotics, chemicals, etc. [3]. Several substances affect the ROS production during the metabolic processes of bacteria, so that it has been observed oxidative balance altered in the presence of different external agents. The stress generated by antibiotics affect bacterial species with different types of metabolism, for example, *Pseudomonas aeruginosa*, a non-fermenting bacteria; *Enterococcus faecalis*, a fermenting microorganism and *Escherichia coli*, a facultative anaerobe [4].

Nitric oxide produced in excess reacts with $O_2^{\bullet-}$ giving rise to the formation of $ONOO^-$, a highly reactive species with capacity for nitration of tyrosine residues, for oxidation of lipids and critical enzymes of the intermediary metabolism, and for breaking DNA chains. Peroxynitrite also oxidizes and give rise to a depletion of endogenous antioxidants, as ascorbate, glutathione, and superoxide dismutase. The progress described contributes a new importance to oxidative stress and to nitrosative stress in the knowledge of the physiological bases of cellular dysfunction [5-7].

The redox reactions of the components of the oxidative stress and nitrosative stress pathways result in the generation of intermediates with different degree of chemical reactivity, i.e., damaging potential. The reaction of $O_2^{\bullet-}$ with NO yields a strong oxidant, $ONOO^-$. The reaction of NO and $O_2^{\bullet-}$ is biologically significant because NO and $O_2^{\bullet-}$ can antagonize each other's biological action, on the other hand, one molecular form of protonated $ONOO^-$, peroxynitrous acid (ONOOH), is a very powerful oxidizing cytotoxic species. Peroxynitrous acid decomposes within a cage that contains two powerful radical species: $HO^{\bullet}$ and nitrogen dioxide (NO_2). The reactivity of these cage-contained radicals towards biomolecules is usually by H abstraction or radical-radical annihilation. In summary, NO has antioxidant and prooxidant properties, in addition to the one-electron redox transitions where it is either reduced to NO^- or oxidized to NO^+. The prooxidant properties are largely represented by its reaction with $O_2^{\bullet-}$ and oxygen (O_2) and yielding $ONOO^-$ and NO_2, respectively. The antioxidant properties of NO are centered on its reactions with metals and with lipid peroxyl radicals in the membrane [5].

Finally, the concept that oxidative metabolism alteration is involved in the mechanism of action of different antibiotics (ATB), it can add the effect of oxidative stress by decreasing the expression of many virulence factors produced by a pathogen that influence host functions and allows the pathogen to grow [8].

According to the above, it is necessary to deepen the understanding of the mechanism of action of antimicrobials to cause stress in bacteria, by both oxygen and nitrogen radicals. Probably, these observations could be used to expose new aspects of antibacterial action, which needs further explanation.

2. MATERIALS AND METHODS

2.1 Antimicrobial Activity Determining the Minimum Inhibitory Concentration and Minimum Bactericidal Concentration

We worked with Gram positive bacteria (*Staphylococcus aureus*) and Gram negative (*Escherichia coli*). Susceptibility testing was performed for assessing the antibacterial ability of a drug based on the inhibition of bacterial growth "in vitro". Measurements of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for each drug were performed according to standards established by the Clinical and Laboratory Standards Institute (CLSI) [9].

In order to quantitatively assess the antimicrobial activity of the microorganism problem faced to a dilution series of ATB under study. The lowest concentration of antibiotic that inhibited bacterial growth after 18 h of incubation was taken as the MIC of drug.

The MBC is defined as the lowest drug concentration able to kill 99.9% in microbial growth compared to the initial inoculum.

We used reference strains of the American Type Culture Collection (ATCC): *S. aureus* ATCC 29213 and *E. coli* ATCC 25922 and clinical isolates of *S. aureus* (235 and 424) and *E. coli* (25 and 30). The bacterial suspensions were obtained from cultures of 18 h in trypticase soy agar (TSA, Britain) or trypticase soy broth (TSB, Britain).

The inoculum was prepared by placing 4 or 5 colonies of each bacterial strain in Mueller Hinton broth (MHB, Britain) compared with the tube turbidity of 0.5 McFarland scale and diluted to adjust the inoculum such that containing $5x10^5$ CFU / mL.

Antimicrobial stock solution was prepared in sterile distilled water. The tests were examined after being incubated 18 h at 35°C and determined the MIC of antimicrobial against the microorganism tested.

To determine the MBC, the tubes where bacterial development was not observed were plated in Mueller Hinton agar (MHA, Britain). Then the plates were incubated 24 h at 35°C. The first plate on which the colony count represented <0.1% of the initial inoculum corresponded to the MBC.

2.2 Antibiotic-induced Oxidative Stress Generation

The production of ROS was determined by the reduction of nitro blue tetrazolium (NBT, Sigma) oxidized (colorless) to its reduced form (NBTH), forming a precipitate (blue formazan). 0.1 mL of bacterial suspension were incubated (OD600 = 1) in phosphate buffer (PBS) pH 7 with 0.1 mL of each drug at concentrations subMIC, MIC and supraMIC, the drug was replaced by PBS in the control samples. Then was added 0.5 mL of 1 mg/mL NBT (SIGMA) and the samples were incubated for 30 min at 37°C. Later was added 0.1 mL of 0.1 N HCl (Cicarelli) to stop the reaction. The tubes were centrifuged 10 min at 1500 rpm to separate the cells from the supernatant (extracellular ROS). The cell pellets were treated with 0.4 mL of dimethyl sulfoxide (DMSO, Cicarelli) to remove the reduced NBT (intracellular ROS) then added 0.8 mL of PBS. The obtained blue formazan was measured by spectrophotometry at 575 nm on a UV-visible spectrophotometer SHIMADZU-160A [4]. Assays were performed in triplicate.

2.3 Determination of Reactive Nitrogen Intermediates in Bacteria

In aqueous solutions, nitric oxide (NO) is converted to nitrite (NO_2^-), therefore, the total concentration of NO_2^- can be used as an indicator of the concentration of ON. Measurements were made by triplicate using a non-enzymatic colorimetric assay using Griess reaction. For the determination of NO in bacteria, were used 18 h cultures of the ATCC strains and clinical isolates of *S. aureus* and *E. coli*. The bacterial suspension was incubated with concentrations subMIC, MIC and supraMIC CIP and Gentamicin (GEN) or in the absence of antibiotic (ATB) (control) for 1 h at 37°C. Then, 0.1 mL of the supernatant was incubated with 50 uL of 2% sulfanilamide in 5% (v/v) HCl and 50 µL of aqueous solution of N-(1-naphthyl) ethylenediamine (NEDD, SIGMA) 0.1%. Azo formation colored derivative was determined after 15 min by spectrophotometry at 540 nm. The absorbance is directly proportional to the concentration of NO_2^- determined by a calibration curve [10].

3. RESULTS

3.1 Antimicrobial Activity Determining the Minimum Inhibitory Concentration and Minimum Bactericidal Concentration

Sensitivity tests determination of the MIC and the MBC are an estimate of the bacteriostatic and bactericidal action of antibiotics. When the MIC of *E. coli* and *S. aureus* to CIP is ≤ 1µg/mL is considered a sensitive bacterial strain, when is equals to 2 µg/mL is considered as intermediate sensitivity and ≥ 4 µg/mL is considered resistant. In the case of *E. coli* and *S. aureus* to GEN, the MIC ≤ to 4 µg/mL is considered a sensitive bacterial strain when is equal to 8 µg/mL is considered as intermediate sensitivity and ≥ to 16 µg/mL is considered resistant (Table 1).

3.2 Antibiotic-induced Oxidative Stress Generation

NBT studies indicated stimulation of oxidative stress with an increase of ROS generated by the antibiotic in the bacterial strains tested.

For CIP, the maximum generation of ROS in susceptible strains of *S. aureus* was obtained with the concentration corresponding to the MIC while the resistant strain of *S. aureus* showed no significant

Table 1. Concentrations selected: subMIC, MIC and supraMIC of Gentamicin and Ciprofloxacin in *S. aureus* and *E. coli*

Strain	Ciprofloxacin			Gentamicin		
	subMIC (µg/mL)	MIC (µg/mL)	supraMIC (µg/mL)	subMIC (µg/mL)	MIC (µg/mL)	supraMIC (µg/mL)
S. aureus ATCC 29213	0.033	0.5	8	0.25	4	64
S. aureus 424	0.033	0.5	8	0.016	0.25	4
S. aureus 235	2	32	512	2	32	512
E. coli ATCC 25922	0.002	0.033	0.5	0.062	1	0.062
E. coli 25	0.0005	0.008	0.125	0.5	8	128
E. coli 30	2	128	512	1	16	256

changes. In strains of *E. coli*, both sensitive and resistant strains did not show significant changes in ROS production over the control without (Fig. 1A), both species GEN treated had a behavior similar to that obtained in the strains of *S. aureus* sensitive to CIP.

The maximum generation of ROS in susceptible strains of *S. aureus* and *E. coli* was obtained from the MIC concentration of ATB and *E. coli* 30 GEN intermediate sensitivity was obtained with supraMIC concentration (Fig. 1B). In susceptible strains of *S. aureus* the increase of ROS was antibiotic concentration dependent. By NBT assay there was generally an increase of ROS in the presence of CIP and GEN (subMIC concentrations, MIC and supraMIC) in the bacterial strains tested.

3.3 Determination of Reactive Nitrogen Intermediates in Bacteria

The production of reactive nitrogen intermediates (RNI) was studied in *S. aureus* and *E. coli* in the presence of CIP and GEN. Figs. 2 and 3 shows the variation of NO_2^- produced by *S. aureus* and *E. coli* incubated with increasing concentrations of the antibiotic.

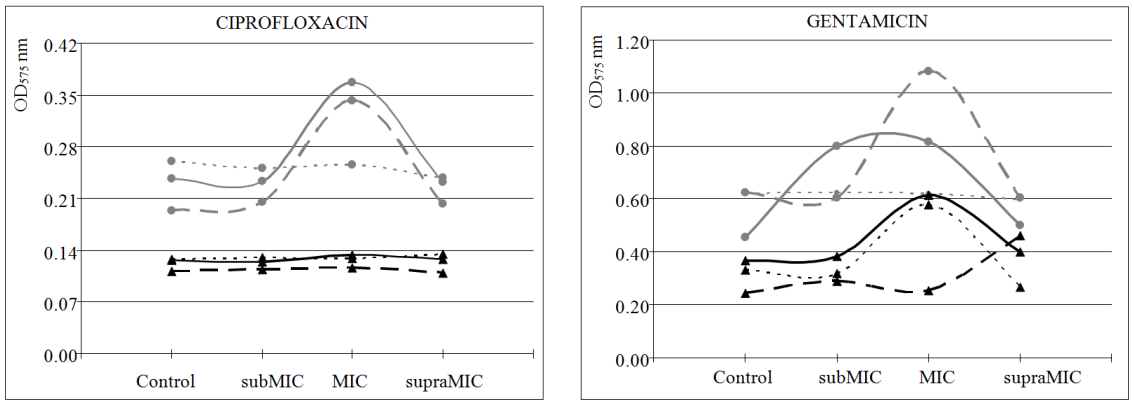
In the presence of CIP-sensitive strains of *S. aureus* 424 and *S. aureus* ATCC, showed a significant increase ($p<0.05$) in the concentration of NO_2^- relative to control without ATB where maximum production of NO_2^- occurred at subMIC concentration (0.033 µg/mL). In the resistant strain to CIP, *S. aureus* 235, no significant changes were observed with the concentrations studied regarding control ATB without ATB (Fig. 2A).

The 3 strains of *E. coli* showed a significant increase ($p<0.05$) in the concentration of NO_2^- relative to control without ATB when they were incubated with CIP (Fig. 2B). In *E. coli* ATCC and *E. coli* 25, the greatest production of NO_2^- concentration occurred at MIC concentration (0.033 µg/mL and 0.008 µg/mL, respectively), whereas in *E. coli* 30 concentration occurred at subMIC concentration (2 µg/mL).

In presence of GEN, the 3 strains of *S. aureus* had a significant increase ($p<0.05$) in the concentration of NO_2^- relative to control without ATB (Fig. 3A). For strains of S. *aureus* ATCC and *S. aureus* 424, the maximum production of NO_2^- occurred at subMIC concentration (0.25 µg/mL and 0.016 µg/mL, respectively) and *S. aureus* 235 occurred at MIC concentration (32 µg/mL).

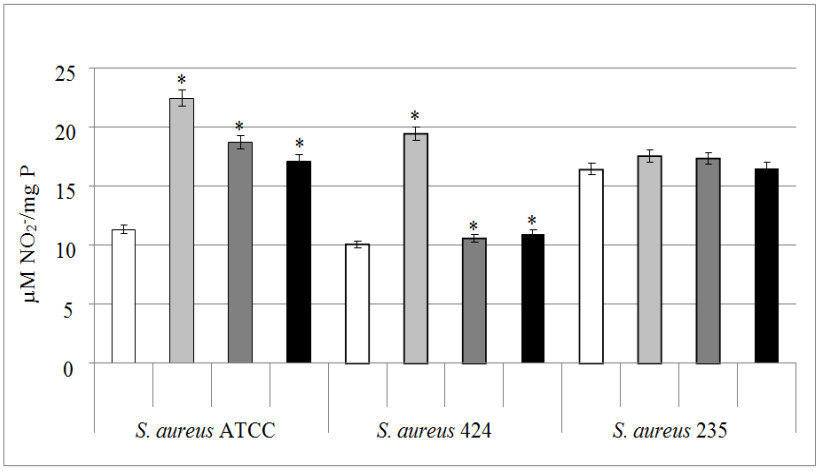
In *E. coli* incubated with GEN the sensitive strain *E. coli* 25 was the only one that showed a significant increase ($p<0.05$) in the concentration of NO_2^- relative to control without ATB (Fig. 3B), the MIC concentration (8 µg/mL) showed the maximum production of NO_2^-.

Similar to ROS generation, we observed an increase in the production of NO_2^- dose-dependent with CIP and GEN. In general, the maximum stimulus of RNI was observed at subMIC concentrations while ROS production was maximal at concentrations corresponding to the MIC. Moreover, it is observed that in the resistant strains, the control level of NO was greater than in sensitive strains.



A **B**

Fig. 1. Determination of ROS in *S. aureus* and *E. coli*. A) In presence of ciprofloxacin at subMIC, MIC, supraMIC concentrations and without ATB (control). B) In the presence of gentamicin at subMIC, MIC, supraMIC concentrations and without ATB (control). *S. aureus* ATCC, *S. aureus* 424, *S. aureus* 235, *E. coli* ATCC, *E. coli* 25, *E. coli* 30



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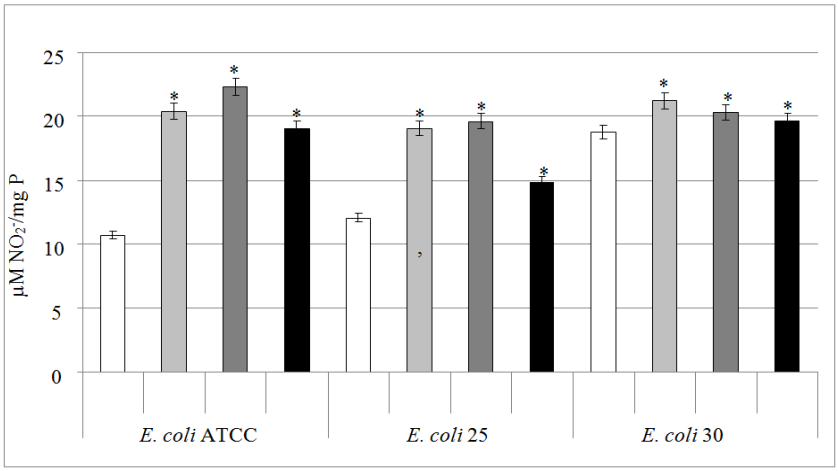


B

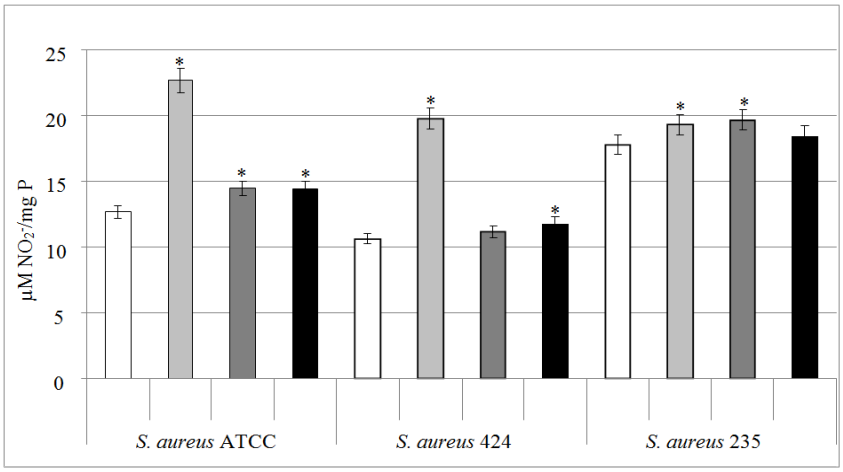
Fig. 2. No determination in presence of CIP. Control □, subMIC ■, MIC ■, supraMIC ■. A) *S. aureus* B) *E. coli.* *p<0.05 respecto del control

To investigate the relationship between ROS and RNI generation, we calculated the ratio ROS/RNI of CIP and GEN at different concentrations.

When *S. aureus* was treated with CIP, the ratio ROS/RNI in sensitive strains *S. aureus* ATCC and *S. aureus* 424 increased relative to control at MIC concentration (0.5 µg/mL), whereas the relationship at supraCIM concentration decreased, this is due to an increase of ROS production with 0.5 µg/mL. In the resistant strain of *S. aureus* 235 the ratio decreased in all concentrations tested compared to the control without ATB since ROS decreased and RNI increased (Table 2).

In presence of GEN, the ratio ROS/RNI in sensitive strains of *S. aureus* ATCC and *S. aureus* 424 increased relative to control without ATB at MIC concentration (4 µg/mL and 0.25 µg/mL, respectively), whereas the relationship at supraMIC concentration decreased, this is due to increased production of ROS at MIC concentrations respect to the production of RNI. In the resistant strain of *S. aureus* 235 the ratio decreased in all concentrations tested compared to the control without ATB, the RNI increased greatly while ROS showed no significant changes (Table 2).

In *E. coli* treated with CIP, the ratio ROS/RNI in the three strains decreased as compared to the control without ATB for all concentrations tested. The RNI increased greater extent than ROS relative to the control, against different concentrations of ATB (Table 3).



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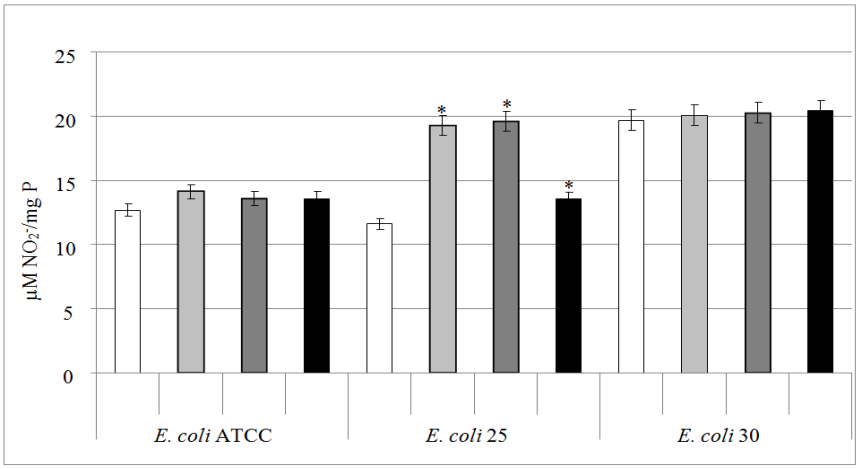

B

Fig. 3. No determination in presence of GEN. Control □, subMIC ■, MIC ■, supraMIC ■. A) *S. aureus* B) *E. coli.* *p<0,05 respecto del control

Table 2. Relation ROS/RNI in ATCC and clinical strains of *S. aureus* incubated with Ciprofloxacin, Gentamicin and without ATB.

Strain	ROS/RNI		
	Sample treatment	CIP	GEN
S. aureus ATCC 29213	Control	0.90	2.62
	subMIC	0.50	1.45
	MIC	0.99	3.99
	supraMIC	0.64	2.24
S. aureus 424	Control	1.23	2.25
	subMIC	0.65	2.18
	MIC	1.82	3.84
	supraMIC	1.11	2.24
S. aureus 235	Control	0.85	1.88
	subMIC	0.77	1.75
	MIC	0.79	1.70
	supraMIC	0.77	1.75

CIP, ciprofloxacin; GEN, gentamicin; ROS, reactive oxygen species; RNI, reactive nitrogen intermediates

Table 3. Relation ROS/RNI in ATCC and clinical strains of *E. coli* incubated with Ciprofloxacin, Gentamicin and without ATB

Strain	ROS/RNI		
	Sample treatment	CIP	GEN
	Control	0.62	1.54
E. coli ATCC 25922	subMIC	0.33	1.44
	MIC	0.32	2.40
	supraMIC	0.36	1.56
	Control	0.56	1.51
E. coli 25	subMIC	0.37	0.90
	MIC	0.35	1.60
	supraMIC	0.48	1.04
	Control	0.32	0.67
E. coli 30	subMIC	0.29	0.78
	MIC	0.31	0.68
	supraMIC	0.30	1.23

CIP, ciprofloxacin; GEN, gentamicin; ROS, reactive oxygen species; RNI, reactive nitrogen intermediates

When *E. coli* was treated with GEN, the ratio ROS/RNI in sensitive strains of *E. coli* ATCC and *E. coli* 25 increased over the control at MIC concentration (1 µg/mL and 8 µg/mL, respectively) whereas, at subMIC and supraMIC concentration, ratio decreased due to increased production of ROS at MIC concentrations related to RNI production. In intermediately susceptible strain of *E. coli* 30, the ratio ROS/RNI increased for all concentrations tested, this is due to an increase of ROS (Table 3).

4. DISCUSSION AND CONCLUSIONS

The results demonstrate that CIP and GEN, independent of mechanism of action, generated an increase of ROS in different bacterial species. To this is added the fact that the stimulation of ROS is immediate, before that the cell death can be possible. Therefore, the generation of high levels of ROS might trigger a series of intracellular events that lead to cell death and accompanying the primary mechanism of action of the ATB [11]. Furthermore, as we observed in previous work [12], by chemiluminescent assays, it was found a higher toxicity with GEN than with CIP in all strains of *E. coli* tested respect to strains on *S. aureus*. Our results suggest that ROS generation contributes to the efficiency of these drugs. Probably, these observations could be used to expose aspects of antibacterial action, which needs further explanation.

When investigating bacterial oxidative metabolism, a greater variation was observed in sensitive strains than in resistant ones, indicating that sensitive strains become more stressed than the resistant strains. This agrees with previous studies which found an increased production of ROS in susceptible strains of *S. aureus* when exposed to CIP, correlating the low MIC values against CIP with increased production of ROS [4]. Based on the above results, it was also observed that GEN generates more ROS than CIP which could be considered the ATB more toxic in the studied species.

Moreover, it was demonstrated that the RNI are also involved in the generation of stress induced by CIP and GEN. Furthermore, it was shown that there is a relationship between the formation of ROS and RNI, coinciding with research conducted in the laboratory where it was found that in the presence of CIP, the ON of resistant strains of *Proteus mirabilis* is consumed by reacting with ROS stimulated by CIP [13]. Cytotoxic properties of a small molecule, lipophilic and freely diffusible as NO are attributed to their high reactivity. Nitric oxide indirect effects are caused by the reaction of this radical with O_2 or $O_2^{\bullet-}$ resulting in the formation of an additional number of RNI. These nitrogen species differ in reactivity, stability and biological activity but result in a broad spectrum of antimicrobial activity. Since the NO consumes $O_2^{\bullet-}$ is suitable for cells a balancing in the ratio of NO and $O_2^{\bullet-}$, or rather between ROS and RNI, as the greatest increase in ROS or RNI consumption leads to increased macromolecules oxidation such as lipids and proteins which brings about a loss of cell viability [14].

The NO ends with oxidative stress through the scavenging of oxidizing species such as $O_2^{\bullet-}$, protects against oxidative stress mediated by the Fenton reaction which involves H_2O_2 and metals (Fe^{2+} y Cu^{+}) and ends the injury caused by $ONOO^{-}$ but form nitrous oxide (N_2O_3) which may result in an increase nitrosative stress. Peroxynitrite generation is maximized when the concentrations of ON and $O_2^{\bullet-}$ are almost equivalent with a small excess of NO, therefore, there must be a fine balance between oxidative and nitrosative stress to maintain a physiological state normal in the cell [15]. According to our results in which the maximum stimulation of RNI was observed at subMIC concentrations of ATB and ROS at concentrations corresponding to the MIC, it appears that, initially the ON generated protects the cell reducing ROS which with increasing concentration of ATB increase the ROS production leading to an imbalance in the ratio of ROS / RNI.

It should be noted that the harmful effect of ATBs in bacteria is irreparable when it affects protein macromolecules, combined with the mechanism of action described for ATBs and oxidative injury of macromolecules which may include changes in membrane protein structural changes leading to increasing classical action described for ATBs [16,17].

Relating to oxidative stress history as part of mechanism of ATB action may be mentioned previous studies in bacteria, which demonstrated that oxidative stress increases susceptibility 70% of the strains to ampicillin and chloramphenicol, 50% of the strains to cloxacillin and tetracycline and 40% of the strains to erythromycin, but does not affect susceptibility to vancomycin and kanamycin [18,19]. Therefore, it is possible that the oxidative and nitrosative metabolic disorder with increased of ROS and RNI could be an important effect that is involved in the mechanism of action of CIP and GEN. Previous research shows that bacteria can become resistant to oxidants and antibiotics simultaneously, suggesting a possible link between oxidative and antibiotic action. [20-24].

The determination of ROS and RNI allowed establish differences in the alteration of oxidative metabolism in susceptible and resistant strains, indicating that

reduced susceptibility to ATBs could relate to less alteration of bacterial oxidative metabolism. In other words, resistance to CIP and GEN may be related to the decreased production of ROS an RNI and consequently, a smaller macromolecules bacterial oxidation. This is related to previous studies that were conducted in the laboratory where it was found that CIP resistance of *P. mirabilis* was associated with a low stimulation of ROS with resultant decrease in the ROS/RNI ratio [13].

The results presented in this work indicate that the oxidative and nitrosative stress caused by CIP and GEN at different concentrations of ATB modifying the balance ROS/RNI in the two bacterial species tested.

Based on the above, we conclude that oxidative stress is associated with the mechanisms of action described for CIP and GEN triggering various effects related to generation of oxidative and nitrosative stress in the cell which lead to bacterial death. This outline coincides with research conducted in the laboratory regarding CIP action on *S. aureus* [25], to which we can now add new aspects related to the bacterial response to oxidative and nitrosative stress in Gram positive and Gram negative bacteria.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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