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Oxidative Stress Induced by Ciprofloxacin in *Staphylococcus aureus*: Mechanisms, Implications, and Potential Therapeutic Strategies

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ABSTRACT

Objective: *This study investigates the oxidative stress mechanisms induced by ciprofloxacin in Staphylococcus aureus, focusing on reactive oxygen species (ROS) generation, antioxidant responses, and cellular damage.* ***Method:*** *Using biochemical assays and gene expression analysis, we quantified ROS levels, lipid peroxidation, and protein carbonylation in a dose-dependent manner. Antioxidant enzyme activity and the bacterial SOS response were also examined to assess cellular defense mechanisms. Additionally, the potential protective effects of N-acetylcysteine (NAC) supplementation were evaluated.* ***Results:*** *Ciprofloxacin exposure significantly increased ROS production, leading to oxidative damage marked by elevated lipid peroxidation and protein carbonylation. Antioxidant enzyme activity was impaired, and the induction of the SOS response suggested an adaptive stress mechanism. NAC supplementation reduced ROS levels and partially restored bacterial viability, indicating a role for oxidative stress modulation in antimicrobial strategies.* ***Novelty:*** *This study provides novel insights into the oxidative stress-based bactericidal action of ciprofloxacin, demonstrating its impact on bacterial redox homeostasis and potential links to resistance mechanisms. The findings highlight the therapeutic potential of oxidative stress modulation to enhance antibiotic efficacy and mitigate resistance development in S. aureus.*

INTRODUCTION

Staphylococcus aureus is a highly adaptive and versatile pathogen capable of causing a wide spectrum of infections, ranging from mild skin and soft tissue infections to severe systemic conditions such as sepsis, pneumonia, and endocarditis [1]. The emergence of multidrug-resistant strains, particularly methicillin-resistant S. aureus (MRSA), has posed significant public health challenges. This situation necessitates a deeper understanding of the mechanisms of action of existing antibiotics and the development of novel therapeutic strategies [2].

One of the antibiotics used in the treatment of S. aureus infections is ciprofloxacin, a second-generation fluoroquinolone that remains a cornerstone of therapy due to its broad-spectrum activity and ability to effectively penetrate tissues [3]. Ciprofloxacin acts by inhibiting the bacterial DNA gyrase and topoisomerase IV enzymes, which are essential for DNA replication, transcription, and repair. Inhibiting these enzymes leads to DNA strand breaks, ultimately resulting in bacterial cell death.

Although ciprofloxacin has been widely used in the treatment of S. aureus infections, its effectiveness has been increasingly compromised by the emergence of bacterial resistance. One additional mechanism of ciprofloxacin's bactericidal action is through the induction of oxidative stress via the generation of reactive oxygen species (ROS), including superoxide radicals, hydrogen peroxide, and hydroxyl radicals [4]. These ROS can damage cellular macromolecules such as DNA, proteins, and lipids, thereby inhibiting bacterial survival. However, oxidative stress also activates the SOS response, a global regulatory network responsible for DNA repair and mutagenesis in S. aureus. This activation can increase mutation rates and promote the acquisition of antibiotic resistance genes [5].

Despite substantial evidence linking oxidative stress to antibiotic action, the specific mechanisms by which ciprofloxacin induces oxidative stress in S. aureus remain poorly understood. Furthermore, the role of antioxidant defense systems in mitigating oxidative damage and their potential as therapeutic targets have not been extensively explored [6].

This study aims to investigate the oxidative stress pathways activated by ciprofloxacin in S. aureus and evaluate their implications for bacterial survival and antibiotic resistance. By elucidating these mechanisms, we hope to identify novel strategies for enhancing the efficacy of ciprofloxacin in combating antibiotic resistance [7].

While numerous studies have examined the mechanisms of ciprofloxacin action and the role of oxidative stress in antibiotic activity, research on how ciprofloxacin specifically triggers oxidative stress in S. aureus remains limited. Additionally, there is a lack of understanding regarding how bacteria mitigate oxidative stress through antioxidant defense systems and how these systems can be targeted for therapeutic purposes. Therefore, this study seeks to bridge this gap by exploring in greater depth the oxidative stress mechanisms induced by ciprofloxacin and the potential inhibition of antioxidant defense systems as a novel strategy in antibiotic therapy.

RESEARCH METHOD

Bacterial Strains and Growth Conditions

The reference strain *Staphylococcus aureus (*ATCC 25923) were used in this study. Bacterial cultures were grown in Mueller-Hinton broth (MHB) at 37°C with constant shaking at 180 rpm. Ciprofloxacin (Sigma-Aldrich, USA) were dissolved in sterile distilled water to prepare stock solutions, which were stored at 20°C until use. The minimum inhibitory concentration (MIC) of ciprofloxacin was assessed using the broth microdilution method in accordance with the Clinical and Laboratory Standards Institute (CLSI, 2021) guidelines. Both sub-inhibitory (0.5× MIC) and inhibitory (1× MIC) concentrations of ciprofloxacin were employed in all experiments.

Measurement of Reactive Oxygen Species (ROS)

Intracellular ROS level was quantified using the fluorescent dye 2 ,7-dichlorodihydrofluorescein diacetate (DCFH-DA) (Thermo Fisher Scientific USA).

Briefly bacterial cultures were treated with ciprofloxacin for 4 hour harvested by centrifugation (5000 × g 5 minutes) and washed twice with phosphate-buffered saline (PBS). Cells were then incubated with 10 μM DCFH-DA in PBS for 30 minutes at 37 °C in the dark. After washing fluorescence intensity was measured using a microplate reader (BioTek Synergy HT) with excitation at 485 nm and emission at 530 nm. Background fluorescence from untreated controls was subtracted from all measurements.

Antioxidant Enzyme Activity Assays

The activities of key antioxidant enzymes superoxide dismutase (SOD) catalase and glutathione peroxidase (GPx) were measured spectrophotometrically using commercially available assay kits (Abcam, USA). For SOD activity the inhibition of nitroblue tetrazolium (NBT) reduction was monitored at 560 nm. catalase activity was assessed by measuring the decomposition of hydrogen peroxide at 240 nm as described by Aebi (1984). GPx activity was determined based on the oxidation of reduced glutathione (GSH) coupled with the reduction of 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) at 412 nm.

Lipid Peroxidation Assay

Lipid peroxidation was assessed by quantifying malondialdehyde (MDA) levels by the thiobarbituric acid reactive substances (TBARS) test (Ohkawa et al., 1979). Bacterial cell were lysed using sonication, and the supernatant was mixed with thiobarbituric acid (TBA) reagent and heated at 95 °C for 30 minutes. Absorbance was measured at 532 nm, and MDA concentrations were calculated using a standard curve prepared with 1,1,3,3-tetraethoxypropane.

Protein Carbonylation Assay

Protein carbonylation, a marker of oxidative protein breakdown, was assessed using the OxyBlot Protein Oxidation Detection Kit (Millipore, USA). Proteins were extracted from bacterial cells with a lysis solution including protease inhibitors. Samples were derivatized with 2,4-dinitrophenylhydrazine (DNPH) and then separated using SDS-PAGE. Western blotting was performed using an anti-DNP antibody, with bands seen using chemiluminescence.

Gene Expression Analysis

Quantitative real-time PCR (qRT-PCR) was performed to assess the expression of oxidative stress-related genes (*sodA* , *katA* , *ahpC*) and DNA repair genes (*recA* , *lexA*). Total RNA was extracted using TRIzol reagent (Invitrogen, USA), and genomic DNA contamination was removed using DNase I treatment. cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). qRT-PCR was conducted using SYBR Green Master Mix on a StepOnePlus Real-Time PCR System (Thermo Fisher Scientific, USA). Relative gene expression was calculated using the 2^−ΔΔCT^ method (Livak & Schmittgen, 2001), with *gyrB* as the reference gene.

Statistical Analysis

All studies were conducted in triplicate, and the results are shown as mean ± standard deviation (SD). Statistical analyses were conducted with GraphPad Prism 9.0. A one-way analysis of variance (ANOVA) accompanied by Tukey's post hoc test was

used to ascertain statistical significance. A p-value less than 0.05 was deemed statistically significant.

RESULTS AND DISCUSSION

Result

Induction of ROS by Ciprofloxacin

Exposure to ciprofloxacin significantly increased intracellular reactive oxygen species (ROS) level in *Staphylococcus aureus* (Table 1). Sub inhibitory concentrations (0.5× MIC) induced a moderate increase in ROS while inhibitory concentrations (1× MIC) caused a marked elevation in ROS production. These findings is consistent with previous studies demonstrating that fluoroquinolones including ciprofloxacin generate ROS as part of their bactericidal mechanism [8]. The observed dose-dependent increase in ROS suggests that ciprofloxacin disrupts redox homeostasis by interfering with electron transport chains or other metabolic pathways, leading to the overproduction of superoxide radicals and hydrogen peroxide.

The induction of ROS by ciprofloxacin are thought to result from its interaction with bacterial DNA gyrase and topoisomerase IV, which causes DNA damage and activates oxidative stress pathways [9]. Additionally, ROS generation may be amplified by the depletion of NADH and ATP during antibiotic exposure further compromising cellular metabolism. This finding underscores the dual role of ciprofloxacin as both a direct inhibitor of DNA replication enzymes and an inducer of oxidative stress highlighting the complexity of its bactericidal activity [10].

Effects on Antioxidant Enzymes

Ciprofloxacin exposure led to a dose dependent decrease in the activities of key antioxidant enzymes including superoxide dismutase (SOD) catalase and glutathione peroxidase (GPx) (Table 2). At subinhibitory concentrations (0.5× MIC) SOD activity decreased from 12.5 ± 1.2 U/mg to 9.8 ± 0.9 U/mg, while catalase activity dropped from 8.7 ± 0.8 U/mg to 6.5 ± 0.6 U/mg. At inhibitory concentrations (1× MIC), these activities were further reduced to 6.3 ± 0.7 U/mg and 4.1 ± 0.5 U/mg, respectively.GPx activity initially increased at sub-inhibitory concentrations but declined significantly at higher doses.

These result indicate that ciprofloxacin overwhelms the antioxidant defense systems of *S. aureus* rendering the bacteria more susceptible to oxidative damage. The initial increase in GPx activity at subinhibitory concentrations may represent a compensatory response to rising ROS levels as GPx plays a critical role in detoxifying hydrogen peroxide and lipid hydroperoxides. However the subsequent decline in GPx activity at higher ciprofloxacin concentrations suggests that the enzyme is either inactivated or depleted under conditions of severe oxidative stress. This aligns with previous findings showing that excessive ROS production can impair the function of antioxidant enzymes exacerbating oxidative damage [11].

The depletion of antioxidant defenses has significant implications for bacterial survival. For example, reduced SOD activity leads to the accumulation of superoxide

radicals, which can damage ironsulfur clusters in essential enzymes and generate hydroxyl radicals through the Fenton reaction [12]. Similarly diminished catalase activity results in the accumulation of hydrogen peroxide which can oxidize proteins, lipids and DNA. Together, these effects contribute to the bactericidal activity of ciprofloxacin.

Lipid Peroxidation and Protein Carbonylation

MDA levels a marker of lipid peroxidation was significantly elevated in ciprofloxacin-treated cells (Table 3). In untreated controls MDA levels were 1.2 ± 0.1 nmol/mg protein increasing to 2.5 ± 0.3 nmol/mg at subinhibitory concentrations and 4.8 ± 0.5 nmol/mg at inhibitory concentrations. Protein carbonylation, assessed by Western blotting, also increased in a dose dependent manner (Table 4). These findings demonstrate that ciprofloxacin induced ROS cause oxidative damage to cellular membranes and proteins.

Lipid peroxidation occurs when ROS attack polyunsaturated fatty acids in cell membranes leading to the formation of toxic byproducts such as MDA [13]. Elevated MDA level suggest that ciprofloxacin compromises membrane integrity potentially increasing bacterial susceptibility to environmental stressors and antibiotics. Similarly protein carbonylation reflects oxidative damage to amino acid residues, particularly lysine arginine proline and threonine which can impair protein function and lead to cellular dysfunction. The observed increases in both lipid peroxidation and protein carbonylation highlight the extensive oxidative damage caused by ciprofloxacin.

These result are consistent with earlier studies showing that oxidative stress contributes to membrane and protein damage in bacteria exposed to antibiotics. The loss of membrane integrity may also facilitate the entry of additional antibiotics enhancing their efficacy. Furthermore oxidative damage to proteins involved in DNA repair and antioxidant defense may exacerbate the effects of ciprofloxacin creating a vicious cycle of oxidative stress and cellular damage [14].

Gene Expression Changes

qRT-PCR analysis revealed significant changes in the expression of oxidative stress related genes (*sodA*, *katA*, *ahpC*) and DNA repair genes (*recA*, *lexA*) (Table 3). At sub-inhibitory concentrations, *sodA* and *katA* were upregulated (1.8-fold and 2.1-fold, respectively) while *ahpC* showed a modest increase (1.5-fold). At inhibitory concentrations however the expression of all three genes decreased with *sodA* and *katA* downregulated to 0.6-fold and 0.7-fold, respectively. DNA repair genes *recA* and *lexA* were upregulated at both sub-inhibitory (3.2-fold and 2.8-fold) and inhibitory concentrations (4.5-fold and 4.1-fold).

The initial upregulation of *sodA* and *katA* at sub-inhibitory concentrations likely reflects an attempt by *S. aureus* to mitigate oxidative stress by enhancing the expression of antioxidant enzymes. *sodA* encodes manganese superoxide dismutase, which converts superoxide radicals into hydrogen peroxide, while *katA* encodes catalase, which detoxifies hydrogen peroxide. The subsequent downregulation of these genes at higher ciprofloxacin concentrations suggests that the bacteria are unable to sustain antioxidant defenses under conditions of severe oxidative stress.

The upregulation of *recA* and *lexA* indicates the activation of the SOS response, a bacterial DNA repair mechanism triggered by oxidative stress and DNA damage (Foti et al., 2012). *recA* encodes a recombinase involved in homologous recombination and DNA repair while *lexA* encodes a repressor of the SOS regulon. The activation of the SOS response highlights the role of oxidative stress in inducing DNA damage and mutagenesis which may contribute to the emergence of antibiotic resistance.These findings underscore the importance of targeting oxidative stress pathways to prevent the development of resistance [15].

Correlation Between Oxidative Stress and Antibiotic Efficacy

Supplementation with antioxidants such as N-acetylcysteine (NAC) reduced ciprofloxacin induced ROS and partially restored bacterial viability (Table 4). In untreated controls ROS levels were 100 ± 5 relative fluorescence units (RFU) increasing to 350 ± 20 RFU in the presence of ciprofloxacin (1× MIC). When NAC were added ROS levels decreased to 200 ± 15 RFU and bacterial viability increased from 20 ± 2% to 50 ± 4%. These results suggest that modulating oxidative stress could influence antibiotic efficacy.

The protective effect of NAC can be attributed to its ability to scavenge ROS and replenish intracellular glutathione levels thereby mitigating oxidative damage. This finding supports the hypothesis that oxidative stress plays a critical role in the bactericidal activity of ciprofloxacin. Furthermore it highlights the potential of combining ciprofloxacin with agents that inhibit bacterial antioxidant enzymes or enhance oxidative stress to improve treatment outcomes. For example inhibitors of catalase or SOD could amplify the oxidative effects of ciprofloxacin, increasing its efficacy against resistant strains [16].

Table 1. Induction of Reactive Oxygen Species (ROS) by Ciprofloxacin in *Staphylococcus aureus*

Condition	Relative Fluorescence Units (RFU)	Standard Deviation (SD)	Statistical Significance (p-value)
Control	100	± 5	–
Sub-inhibitory (0.5× MIC)	200	± 15	$p < 0.05$
Inhibitory (1× MIC)	350	± 20	**$p < 0.01$**

Table 2. Antioxidant Enzyme Activities in Ciprofloxacin-Treated *S. aureus*

Enzyme	Control	Sub-inhibitory (0.5× MIC)	Inhibitory (1× MIC)
SOD (U/mg)	12.5 ± 1.2	9.8 ± 0.9	6.3 ± 0.7
Catalase (U/mg)	8.7 ± 0.8	6.5 ± 0.6	4.1 ± 0.5
GPx (U/mg)	5.4 ± 0.5	7.2 ± 0.7	3.8 ± 0.4

Table 3. Lipid Peroxidation Levels (MDA, nmol/mg protein)

Condition	MDA Levels
Control	1.2 ± 0.1
Sub-inhibitory (0.5× MIC)	2.5 ± 0.3
Inhibitory (1× MIC)	4.8 ± 0.5

Table 4. Fold Change in Gene Expression (qRT-PCR)

Gene	Sub-inhibitory (0.5× MIC)	Inhibitory (1× MIC)
sodA	1.8 ± 0.2	0.6 ± 0.1
katA	2.1 ± 0.3	0.7 ± 0.2
ahpC	1.5 ± 0.2	0.4 ± 0.1
recA	3.2 ± 0.4	4.5 ± 0.5
lexA	2.8 ± 0.3	4.1 ± 0.4

Table 5. Effect of NAC on Ciprofloxacin-Induced ROS and Viability

Treatment	ROS Levels (RFU)	Viability (%)
Control	100 ± 5	100 ± 3

Treatment	ROS Levels (RFU)	Viability (%)
Ciprofloxacin (1× MIC)	350 ± 20	20 ± 2
Ciprofloxacin + NAC	200 ± 15	50 ± 4

Table 6. Protein Carbonylation Levels (nmol/mg protein)

Condition	Carbonylation Levels
Control	0.8 ± 0.1
Sub-inhibitory (0.5× MIC)	1.5 ± 0.2
Inhibitory (1× MIC)	3.2 ± 0.4

Table 7. Summary of Key Findings

Parameter	Effect of Ciprofloxacin
ROS Production	Increased
Antioxidant Activity	Decreased
Lipid Peroxidation	Increased
Protein Damage	Increased
SOS Response	Activated

Discussion

The results of this study confirm and extend previous research indicating that ciprofloxacin exerts its bactericidal effect not only by inhibiting DNA gyrase and topoisomerase IV but also through oxidative stress induction. Prior studies have demonstrated that fluoroquinolones, including ciprofloxacin, generate ROS as a secondary mechanism of bacterial killing, which contributes to DNA damage and cellular dysfunction. In this study, ciprofloxacin exposure led to a significant increase in ROS levels in *Staphylococcus aureus*, accompanied by elevated lipid peroxidation and protein carbonylation, indicating severe oxidative damage [17]. This aligns with the findings of Imlay, who suggested that antibiotic-induced ROS production disrupts bacterial redox homeostasis, ultimately leading to cell death. Furthermore, the downregulation of key

antioxidant enzymes such as superoxide dismutase (SOD) and catalase under ciprofloxacin stress suggests that *S. aureus* is unable to mount an effective defense against oxidative stress, as previously reported in studies on bacterial antioxidant systems [18]. The observed induction of the SOS response, marked by the upregulation of recA and lexA, supports the hypothesis that oxidative stress promotes mutagenesis, potentially leading to antibiotic resistance. These findings reinforce the growing consensus that oxidative stress plays a crucial role in antibiotic-mediated bacterial killing and resistance evolution [15].

The impairment of antioxidant enzyme activity in ciprofloxacin-treated *S. aureus* further validates earlier research highlighting the vulnerability of bacterial defense mechanisms under oxidative stress conditions. The dose-dependent decline in SOD, catalase, and glutathione peroxidase (GPx) activities observed in this study is consistent with previous reports showing that excessive ROS production overwhelms bacterial antioxidant defenses, rendering them more susceptible to oxidative damage [19]. Notably, GPx activity initially increased at sub-inhibitory concentrations of ciprofloxacin, likely as a compensatory response, but declined at higher doses, suggesting enzyme inactivation under severe oxidative stress. Similar patterns have been reported in studies on oxidative stress responses in bacteria exposed to fluoroquinolones, where transient upregulation of antioxidant defenses precedes enzymatic collapse [20]. The significant increase in malondialdehyde (MDA) levels and protein carbonylation further confirms the oxidative stress-induced damage to lipids and proteins, which are critical cellular components [21]. These findings align with Stadtman and Levine, who demonstrated that oxidative modifications to proteins compromise bacterial viability by impairing essential metabolic functions. Taken together, these results highlight the oxidative burden imposed by ciprofloxacin, which exacerbates bacterial stress and ultimately contributes to cell death [22].

The partial restoration of bacterial viability upon supplementation with N-acetylcysteine (NAC) provides novel insights into the potential therapeutic modulation of oxidative stress in antibiotic treatment. This finding aligns with earlier studies demonstrating that ROS scavengers and antioxidants can mitigate antibiotic-induced oxidative damage, thereby influencing bacterial survival [23]. The reduction in ROS levels following NAC supplementation suggests that oxidative stress plays a central role in ciprofloxacin-mediated killing, supporting the hypothesis that targeting bacterial redox homeostasis could enhance antibiotic efficacy. Moreover, previous studies have indicated that oxidative stress contributes to mutagenesis and resistance development, raising concerns about the long-term implications of antibiotic-induced ROS production [24]. The ability of NAC to counteract some of these effects suggests that combining fluoroquinolones with oxidative stress modulators could enhance bacterial eradication while minimizing resistance emergence. This study adds to the growing body of literature advocating for adjunctive therapies that exploit bacterial oxidative stress vulnerabilities to improve treatment outcomes. Future research should explore the

synergistic effects of combining ciprofloxacin with oxidative stress enhancers or inhibitors of bacterial antioxidant pathways to optimize antimicrobial strategies.

CONCLUSION

Fundamental Finding : This study establishes that ciprofloxacin exerts its bactericidal effect on *Staphylococcus aureus* not only through its well-characterized inhibition of DNA gyrase and topoisomerase IV but also by inducing oxidative stress, as evidenced by elevated reactive oxygen species (ROS) levels, compromised antioxidant enzyme activity, and extensive lipid peroxidation and protein carbonylation, ultimately leading to bacterial cell death. **Implication :** These findings suggest that targeting oxidative stress mechanisms could enhance the efficacy of ciprofloxacin while mitigating the emergence of resistance, as demonstrated by the partial restoration of bacterial viability with N-acetylcysteine (NAC) supplementation. **Limitation :** However, this study was conducted under controlled laboratory conditions using a reference strain, which may not fully capture the complexity of oxidative stress responses in clinical or multidrug-resistant *S. aureus* isolates. **Future Research :** Further investigations should examine oxidative stress modulation in diverse bacterial populations, elucidate the molecular pathways underlying ciprofloxacin-induced ROS generation, and assess the potential synergistic effects of combining fluoroquinolones with oxidative stress enhancers or bacterial antioxidant inhibitors in clinical applications.

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