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Antioxidant-Mediated Modulation of Bacterial Antibiotic Susceptibility

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Abstract: Background: Fluoroquinolones like Ciprofloxacin are broad-spectrum antibiotics. They act on targeted bacteria by interfering with DNA replication, leading to the generation of free radicals which ultimately contribute to bacterial death. Conversely, dietary antioxidant supplements are commonly prescribed during the course of infection treatment. Co-administration of a multivitamin preparation alongside fluoroquinolones might play a role in fluoroquinolone treatment failure. **Objectives:** Thus, the present study aimed to investigate the effect of antioxidants like vitamin E, vitamin C, and vitamin B12 on the action of ciprofloxacin against various standard bacterial strains and ciprofloxacin-sensitive clinical isolates. **Methods:** The antibacterial activity of ciprofloxacin in the absence and presence of these antioxidants was assessed using the Kirby-Bauer disc diffusion method and by determining the minimum inhibitory concentration. **Results:** All strains tested in the study demonstrated sensitivity to ciprofloxacin alone. However, when vitamin C, E, or B12 was incorporated into the test medium, all strains exhibited significantly smaller zones of inhibition and elevated MIC ranges compared to ciprofloxacin alone. **Conclusion:** The generation of reactive oxygen species during the antibacterial action of ciprofloxacin on bacterial cells was either diminished or attenuated after supplementing the media with vitamin C, E, or B12. These antioxidants acted as free radical scavengers, protecting bacterial cells from the effects of generated free radicals. As the presence of antioxidants in the environment modulates the action of ciprofloxacin, the ingestion of these antioxidants should be closely monitored during the treatment course.

Key Words: ischemia reperfusion; etanercept; NF-kB; TLR

I. INTRODUCTION

Fluoroquinolones, such as ciprofloxacin, are synthetic broad-spectrum antibacterial agents active against both gram-positive and gram-negative bacteria. Ciprofloxacin is a potent drug for combating various infections caused by different bacteria, yielding excellent results with relatively fewer adverse effects [1]. Its mechanism of action involves interference with DNA replication and transcription by inhibiting bacterial DNA gyrase/topoisomerase II and IV. This results in the formation of quinolone-enzyme-DNA complexes (cellular poisons), triggering the generation of oxidative free radicals. These radicals subsequently increase cellular toxicity and contribute to cell death. During this antimicrobial action of ciprofloxacin, major reactive oxygen species formed include singlet oxygen ($^1O2$) and superoxide anion (O2-) [2].

Dietary supplements, such as vitamin C (ascorbic acid), vitamin E (tocopherol), and vitamin B12 (cobalamin), are micronutrients that play crucial roles in various biological processes within the human body, making them essential [3]. Additionally, these vitamins act as antioxidants, working to prevent the formation of free radicals and inhibiting their reactions with biological structures [4]. Vitamin E serves as an essential lipid-soluble antioxidant by scavenging hydroperoxyl radicals [5]. As an antioxidant, vitamin C protects against oxidative stress-induced cellular damage by scavenging reactive oxygen species (ROS) [6]. Furthermore, vitamin B12 has shown a potential protective role against toxicity induced by chemicals or drugs. Supplementation with vitamin B12 has been found to significantly reduce oxidative DNA damage and enhance DNA repair mechanisms [7].

These dietary supplements, possessing antioxidant properties, are frequently prescribed by clinicians alongside antibiotics during infection treatment [6]. Moreover, instances of fluoroquinolone treatment failure have been reported among

elderly patients who were concurrently taking multivitamin supplements [8].

Hence, it becomes crucial to understand the effects of these antioxidants on the antibacterial action of antibiotics. In the present study, we investigated the impact of three major antioxidants-namely, vitamins E, C, and B12-on the antibacterial activity of ciprofloxacin against a range of standard strains as well as clinical isolates. This study delved into the modulation of bacterial antibiotic susceptibility mediated by antioxidants.

II. MATERIALS AND METHODS

This prospective analytical study was conducted in the Department of Microbiology over a three-month period. The research aimed to investigate the potential interference of antioxidants with the antibacterial action of ciprofloxacin against standard reference bacteria and ciprofloxacin-sensitive clinical isolates.

BACTERIAL CULTIVATION AND GROWTH CONDITIONS

All standard reference strains and clinical isolates were stored at -20°C in trypticase soy broth with 20% glycerol (Hi Media, India). For batch susceptibility testing, samples were thawed and utilized as needed.

ANTIBIOTIC SUSCEPTIBILITY TESTING

Antibiotic susceptibility testing was performed for all strains using Mueller-Hinton (MH) agar (Hi Media) and 30 μg ciprofloxacin discs (Hi Media). The test was conducted using the Kirby-Bauer disc diffusion method following the guidelines of the Clinical and Laboratory Standards Institute (CLSI) [9]. Isolated bacterial colonies were obtained on nutrient agar. A colony was suspended in sterile saline to create a homogenous suspension with turbidity matching a 0.5 McFarland standard. Results for each isolate were recorded after 18 hours of incubation by measuring the zone of complete inhibition around the ciprofloxacin disc. Additional experiments were performed by supplementing MH agar separately with vitamin E, vitamin C, and vitamin B12 at final concentrations of 100 mM, 10 mM, and 100 μM, respectively [10], [11]. These results were used as a qualitative measure to comprehend differences in sensitivity patterns with ciprofloxacin alone and in the presence of the mentioned antioxidants. Each experiment was repeated three times for reproducibility, and the mean of three independent experiments was recorded.

DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION (MIC)

MIC for ciprofloxacin was determined using the agar dilution method in accordance with CLSI guidelines [9]. Briefly, ciprofloxacin was serially diluted and incorporated into molten MH agar. Bacterial suspensions with approximately 10^5 CFU were prepared from pure isolated colonies of each strain, with turbidity matching a 0.5 McFarland standard.

To assess ciprofloxacin's activity in the presence of antioxidants (vitamin C, E, and B12), independent experiments were performed by incorporating the antioxidants in molten MH agar along with serially diluted ciprofloxacin. The final concentrations of vitamin C, E, and B12 in MH agar were 100 mM, 10 mM, and 100 μM, respectively. All media were poured, dried, and tested for sterility before being used for determining MIC. Inoculums were spotted using a micropipette delivering 10 μl per spot. After 18 to 20 hours of incubation at 37°C, plates were examined. MIC was defined as the lowest concentration of ciprofloxacin preventing visible growth. Slight hazes of growth or colonies fewer than three were disregarded [9]. Plates were read in duplicates, and the highest values were recorded. Breakpoint drug concentrations to determine resistance or susceptibility were determined following CLSI guidelines [9].

III. STATISTICAL ANALYSIS

The antibiotic susceptibility testing using the Kirby-Bauer disc diffusion method was conducted in triplicates to assess result reproducibility. All data obtained from these experiments were presented as means $\pm$ SD. The determination of MIC for ciprofloxacin was simultaneously performed for both standard control strains and clinical isolates, and the results were compared. The obtained results were analyzed using Student's t-test, with a significance level of $P < 0.05$ considered statistically significant.

IV. RESULTS

In this research, the interaction and effects of three different compounds, namely vitamin C, E, and vitamin B12, with the antibacterial action of ciprofloxacin, were investigated against various clinical isolates and standard reference strains. A total of 36 consecutive ciprofloxacin-sensitive strains were obtained from routine clinical specimens in the bacteriology section. Among these strains, 16 isolates were from urine, 14 from pus, two from sputum, three from blood, and one isolate was from stool. All strains were identified up to the species level following standard recommended procedures [12]. The isolated bacteria were identified as Escherichia coli (16), Coagulase Positive Staphylococci (COPS) (10), Pseudomonas aeruginosa (5), and Klebsiella pneumoniae (5). Additionally, standard reference strains including Escherichia coli ATCC 35218, Staphylococcus aureus ATCC 29213, Pseudomonas aeruginosa ATCC 9027, and Klebsiella pneumoniae ATCC 13883 were used as controls and processed concurrently.

Results from the disc diffusion test revealed that ciprofloxacin induced antibacterial action against all strains. Each strain displayed a zone of inhibition measuring $\geq$15 mm around the ciprofloxacin disc, indicating susceptibility [11].

However, susceptibility testing for all standard strains performed on MH agar supplemented with either vitamin C, E, or B12 showed reduced zones of inhibition around the ciprofloxacin disc (Figure 1). The present study's results

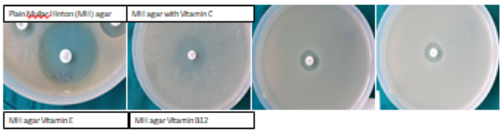

FIGURE 1: Zones of inhibition of ciprofloxacin (30 ug disks) alone and ciprofloxacin in presence of Vit. C, E and B12

demonstrated that the combination's zones of inhibition were significantly smaller than those of ciprofloxacin alone for all standard strains (Table 1). Similar results were also observed when testing the remaining 36 clinical isolates simultaneously.

Difference between zones of inhibition for Ciprofloxacin alone and in combinations with antioxidants for all strains was highly significant (P<0.0001).

The zone of inhibition for ciprofloxacin alone was significantly ($P < 0.05$) smaller than those of the combination of ciprofloxacin with vitamin C, E, and B12 for all standard bacterial strains. The results are presented as the mean $\pm$ SD of three independent experiments.

The minimum inhibitory concentration (MIC) of ciprofloxacin alone and in combination with these antioxidants was also determined. Supplementation with vitamin C, E, or Vitamin B12 substantially reduced the antibacterial activity of ciprofloxacin. This was indicated by significantly higher MIC values obtained in the presence of these antioxidants compared to the MIC values with ciprofloxacin alone (Table 2).

The MIC values for ciprofloxacin alone were significantly ($P < 0.05$) lower than those of the combination of ciprofloxacin with vitamin C, E, and B12 for all clinical isolates.

A marked reduction in the zone of inhibition was noted in the presence of vitamin C, followed by vitamin E and vitamin B12, compared to their corresponding controls (Figure 1). The action of ciprofloxacin on all bacteria was visibly reduced in the presence of these antioxidants. This indicates that all antioxidants play a role in the antibacterial action of ciprofloxacin and provide protection to bacteria in the presence of antibiotics. The qualitative estimation of the protection offered by these antioxidants to the bacteria was determined by the MIC of ciprofloxacin. We observed similar results, with a significant increase in the MIC values of ciprofloxacin in the presence of vitamin C, followed by vitamin E and vitamin B12 (Figure 2).

MIC values for ciprofloxacin alone were significantly ($p < 0.05$) lower than those of the combination of ciprofloxacin with vitamins C, E, and B12 for all standard bacterial strains.

V. DISCUSSION

The overall aim of the present study was to understand the effect of antioxidants on the antibacterial efficacy of ciprofloxacin when tested against a wide range of bacteria.

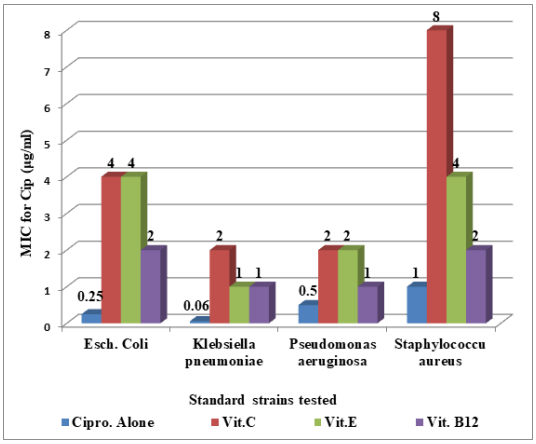

FIGURE 2: Comparison between MIC of ciprofloxacin alone and ciprofloxacin in presence of Vit. C, E and B12 against standard strains

The results of the present study indicated that simultaneous use of ciprofloxacin along with antioxidants such as vitamins E, C, and B12 resulted in a marked reduction in the antibacterial activity of ciprofloxacin against various standard control strains and clinical isolates. We report reduced sensitivity of ciprofloxacin in all the assays against all the strains in the presence of these vitamins. Marked reduction in the zone diameter and significantly elevated MIC values clearly indicate the role of these vitamins against the bactericidal action of ciprofloxacin. Thus, the present study shows that the concurrent use of ciprofloxacin along with antioxidant supplementation might have negatively interacted with the antibacterial action of ciprofloxacin.

It is a well-known fact that the bactericidal action of ciprofloxacin is exerted by interfering with DNA replication through the inhibition of DNA gyrase and DNA topoisomerase II [13]. Ciprofloxacin also induces its antibacterial action via the generation of reactive oxygen species (ROS), which contributes to cell death following gyrase poisoning [14]. It has also been reported that in the presence of ciprofloxacin, sensitive bacteria showed elevated levels of intracellular superoxide compared to the resistant ones. These increased levels of intracellular free radicals eventually contribute to cell death [15]. In the present study, the supplementation of exogenous antioxidants might have led to the attenuation of the generation of these free radicals, where they acted as ROS scavengers. Thus, the presence of these ROS scavengers in the environment offers protection to bacteria from the bactericidal action of ciprofloxacin. This study further emphasizes the importance of reactive oxygen species for the antibacterial action of ciprofloxacin in a variety of bacterial strains.

Our results are in accordance with previous work, where it has been shown that the incorporation of glutathione and vitamin C reduced the antibacterial activity of ciprofloxacin against *E. coli* [16]. Gene knockout studies reported that this reduction was dependent on free radical scavenging [16].

Standard strains	Zones of inhibition (mm)			
	Ciprofloxacin	Cipro+Vit C	Cipro+Vit E	Cipro+Vit B12
Esch coli ATCC 35218	27.2 ±0.6	14.2 ±0.6	17.2 ±0.6	18.2 ±0.6
P. aeruginosa ATCC 9027	31.0 ±1.0	17±0.6	24.2 ±0.6	26.2 ±0.6
Kleb. pneumoniae ATCC 13883	32.3 ±0.6	19.2 ±0.6	23.2 ±0.6	25.2 ±0.6
S. aureus ATTC 29213	21.2 ±1.0	12.4 ±0.6	15.2 ±0.6	18.2 ±0.6

TABLE 1: Comparison between the zone of inhibition of ciprofloxacin and ciprofloxacin in presence of Vitamin C, E and B12, against standard strains. Results were presented as Mean SD of three independent experiments

Microorganisms	Ciprofloxacin alone and with antioxidants	Number of strains having MIC (μg/ml)							
		≥8	4	2	1	0.5	0.25	0.125	0.062
E. coli (n=16)	**CIP alone**	0	0	0	7	2	1	1	5
	CIP+Vit E	1	4	3	3	2	2	1	0
	CIP+Vit C	2	2	5	1	3	3	0	0
	CIP+Vit B12	4	3	2	0	3	1	3	0
K. pneumoniae (n=5)	**CIP alone**	0	0	0	2	0	0	1	2
	CIP+Vit E	0	1	1	1	2	0	0	0
	CIP+Vit C	0	1	2	0	2	0	0	0
	CIP+Vit B12	0	2	0	0	0	2	1	0
P. aerugionosa (n=5)	**CIP alone**	0	0	0	1	0	2	1	1
	CIP+Vit E	0	0	1	3	1	0	0	0
	CIP+Vit C	0	1	2	1	0	1	0	0
	CIP+Vit B12	0	0	1	4	0	0	0	0
COPS (n=10)	**CIP alone**	0	0	0	6	2	2	0	0
	CIP+Vit E	1	5	0	4	0	0	0	0
	CIP+Vit C	2	2	3	3	0	0	0	0
	CIP+Vit B12	2	3	3	1	1	0	0	0

TABLE 2: Comparison between MIC of ciprofloxacin alone and ciprofloxacin in presence of Vit. C, E and B12 against clinical isolates

Previous reports have also shown that α-tocopherol protects cultured fibroblasts against ciprofloxacin-induced oxidative stress [17]. Furthermore, vitamin B12 supplementation prevented DNA damage induced by a number of drugs via its scavenging action against oxidative DNA damage [18]. In another study, the antibacterial action of ciprofloxacin and levofloxacin was found to be significantly inhibited when they were combined with vitamin B12 [11].

Results of this study showed that the protective action against ciprofloxacin was more pronounced with vitamin C followed by vitamin E and vitamin B12. This is indicated by the significantly smaller diameters of the zone of inhibition for ciprofloxacin in the presence of vitamin C compared to vitamin E and vitamin B12. In correlation with that, the MIC values in the presence of vitamin C were several folds higher than those of other antioxidants used.

This study has some limitations. In the present work, only standard strains and a limited number of clinical isolates have been utilized. The present study shows that the presence of antioxidants rescues bacteria against the antibacterial action of ciprofloxacin; however, further investigations are required to understand the exact mechanism of this phenomenon with a wide range of bacteria and other fluoroquinolones as well.

The wide use of ciprofloxacin and its significant therapeutic value makes these observations noteworthy. These findings are especially important when an antibiotic like ciprofloxacin is prescribed to patients with infections, particularly those who are also on antioxidant supplementation. Therefore, to balance these opposing reactions, the ingestion of these vitamins might need to be closely monitored or adjusted in patients who are undergoing ciprofloxacin treatment.

VI. CONCLUSION

In conclusion, marked inhibition was noted against the antibacterial action of ciprofloxacin when it was combined with antioxidants, namely Vitamin E, C, and B12. The significance of this observation arises from the widespread use of these antioxidants and the high therapeutic value of commonly used fluoroquinolones like ciprofloxacin. Thus, investigations into clinical consequences as well as close monitoring of patients being treated for bacterial infections are recommended.

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