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Effect of Vitamin C on Penicillin G Efficacy to Inhibit Bacterial Populations

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ABSTRACT

Bacterial resistance to antibiotics may have evolved from either the natural resistance mechanism associated with bacterial structure and/or related to various bacterial strategies, ranging from the enzymatic inactivation of the antibiotic, to drug efflux and to the development of multidrug resistance clones. There is increasing interest in some vitamins, such as Vitamin C, as potential agents for reducing bacterial persistence and, therefore, these may help in treating many infectious diseases of bacterial origin.

This study was designed to determine the possible effect of Vitamin C in inactivating bacterial populations of *Staphylococcus aureus* and *Escherichia coli*, alone and in combination with Penicillin G. Potential antibacterial effect was evaluated using the Cup Cut Agar diffusion method. Inoculated with about 1x108 cfu.ml^{-1} from a 16 to 18 hr culture of bacteria. A Vitamin C concentration, calculated to 10 mg, was added to a range of Penicillin G concentrations of 30, 60 and 120 mg. Plates were then incubated overnight at 37^{o}C. All experiments were performed in 5 replicates, where the mean was used to extrapolate our data.

The results showed that Vitamin C displayed a similar antibacterial effect to that of Penicillin G (at 120 mg) on *E. coli* populations where this effect was significantly ($P < 0.05$) enhanced with the three Penicillin G concentrations employed. *S. aureus* responded to the effect of the combined Vitamin C and Penicillin G more than *E. coli*. Therefore, Vitamin C potentiated the effect of the Penicillin G antibiotic to an extent that the combined sub-effective or sub-therapeutic dose of Penicillin G (30 mg) with Vitamin C (10 mg) displayed a similar effect to that of 120 mg Penicillin G alone.

It may be elucidated that Vitamin C facilitates access of the treatment agent to its target, resolving some aspects of the problem of bacterial resistance. This study provides evidence that Vitamin C displays a crucial role in inactivating bacterial populations and has the ability to maximize the effect of some antibiotics deployed at a lower concentration that might help in minimizing some undesirable side effects. Vitamin C, in addition to its role in maintaining human immunity against the cold virus and its antioxidant role can, therefore, contribute to the treatment of bacterial infectious diseases that are non-responsive to a variety of antibiotics.

Keywords - *Penicillin G; Vitamin C; Resistance; Persistence; Antibacterial.*

INTRODUCTION

The use and application of antibiotics to treat infections is always associated with problems displayed by the microorganism. Such problems are expressed in the form of bacteria being resistance to these agents or the failure of these microorganisms to totally succumb to the effect of these treatments. The phenomenon of the inability of antibiotics to kill bacterial populations has been reported for several decades. For example, in the early 1940's, the physician Bigger discovered that a fraction of *Staphylococci* had persisted to any further kill by the antibiotic penicillin.[1] Importantly, bacterial resistance has appeared for every major class of antibiotic.[2] Since the introduction of antibiotics, the emergence of bacterial resistance has become increasingly evident, particularly for important pathogens such as *Escherichia coli*, *Salmonella* spp., *Campylobacter* spp., *Enterococcus* spp. and *Staphylococcus* spp.[3,4]

In this context, research has focused on various approaches to reduce the risk of generating such persistence/resistance fractions.[5-14] In such instances, researchers have made many trials to use supportive material that could be of natural origin or natural products, including human dietary components, to support the action of antibiotics and antibacterial agents to disable bacteria from causing disease. Antibiotics have been thoroughly investigated as they are frequently prescribed to cure diseases of bacterial origin. The use of these agents, therefore, is aimed to be selective at their therapeutic concentration in inactivating the pathogenic bacteria while preserving the human microbiota.[15]

A number of antibiotics that attack bacteria through interfering with the cell wall function include the Beta-Lactam antibiotic Penicillin. It inactivates bacteria through interfering with the synthesis of peptidoglycan, leading to

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death and lysis. Intuitively, this type of antibiotic requires the bacteria to be in the growing phase to exert it's action as resting bacteria display no signs of peptidoglycan biosynthesis.[9] Penicillin acts against both Gram positive and Gram negative bacterium, to various extents, as far as the microorganism is of the penicillinase-negative type. These include:

i) *S. aureus,* widely found, known to cause a number of diseases; for example, boils and abscesses, impetigo, meningitis, osteomyelitis, pneumonia, septic phlebitis and endocarditis[16-19] and

ii) some species of Gram-negative bacilli, previously considered susceptible to very high intravenous doses of Penicillin G (up to 80 million units/day), including some strains of *Escherichia coli*, *Proteus mirabilis*, *Salmonella* and *Shigella* spp.[20,21]

The use of some daily food supplements may have some positive effect on the antibacterial activity of antibiotics in addition to their basic effect. Vitamin C, as a part of the daily human diet, plays a very important role as an antioxidant agent. It prevents a number of oxidation-related disorders, such as cancer[22,23] (by preventing the formation of carcinogens from precursor compounds), Alzheimer's disease, atherosclerosis and some degenerative diseases.

The concept of Vitamin C possessing a potential role in helping the human body to overcome problems of bacterial infections, has evolved within the local community.[24] This may partially be explained by Vitamin C assisting the antibacterial action of the antibiotic in inactivating the bacteria[25], either through facilitating the access of the agent to its target, or in turn, Vitamin C itself may have a negative effect on the bacterial survival. In this study, the authors aim to investigate the effect of Vitamin C on the susceptibility of both *S. aureus* and *E. coli* towards the Penicillin G antibiotic using the cup-cut agar diffusion method.

MATERIALS AND METHODS

Chemicals and reagents

Penicillin G sodium pure powder used throughout this study was obtained from Sandoz Pharmaceuticals Co., Kundl-Austria. Vitamin C was obtained from Akmus CO., India. Nutrient broth, Nutrient agar, Muller-Hinton agar and other media were used to grow wild type strains. All dehydrated media were obtained from BDH (Poole, U.K.). Iodine, Phosphate Buffer Saline (used to prepare stock solutions of the antibiotic) and glycerol were obtained from Sigma Chemicals Co. (Poole U.K.). All reagents were of the highest grade of purity (Aristar) from the BDH suppliers.

Microorganism and culture maintenance

S. aureus (ATCC 29213) and *E. coli* (ATCC 25922) strains were maintained in a long term cryogenic system. This was prepared in the lab using 10% v/v glycerol in nutrient broth media. The stock was dispensed into 1 ml aliquots and stored at -80°C till required. Fresh cultures were prepared from frozen stocks prior to every experimental procedure. This was done by streaking, using a sterile loop, onto freshly prepared nutrient agar plates which were then incubated at 37°C for 18 hr, from which 5 to 6 colonies were used to run the experiment.

Experimental methods

i) Iodometric test for Beta-lactamase enzyme detection

The beta-lactamase producing *S. aureus* type is determined by using the Iodometric technique for the detection of the beta lactamase enzyme.[26,27] This was conducted by adding a few drops of concentrated iodine to a suspension of test bacteria made up from 1 ml, of 18 hr culture prepared from 5-6 colonies, in sterile saline. Reducing the colour of the bacterial suspension with the iodine from blue to colourless within 10 min, indicated that the bacteria is a Beta-lactamase producing bacteria.

ii) Antimicrobial susceptibility sxperiments

The antimicrobial activity experiments of Penicillin G and Vitamin C were performed using the cup-cut agar diffusion technique.[28-30] This was performed by swabbing pre-dried sterile plates of Nutrient agar and Muller-Hinton agar with a specific inoculum of test bacteria, collected from late exponential phase culture, prepared in sterile saline. This corresponded to $1x10^8$ cfu.ml^{-1} at an absorbance of 470 nm of 1.2. Specific concentrations of the antibiotic Penicillin G (30, 60 and 120 mg.ml^{-1}) prepared in PBS were used. Vitamin C was used at a concentration of 10 mg.ml^{-1} throughout. All experiments were performed alongside controls (PBS). The volume of all test solutions was 0.1 ml. All plates were incubated at 37°C for 24 hr and then examined to determine the presence and extent of the zone of inhibition. All experiments were repeated 6 times and the mean was used to extrapolate our data.

Statistical analysis

Statistical analysis was conducted using SPSS (software packing version 13). Descriptive statistical analysis was applied; the non-parametric Kolmogorove-Smirnov maximum deviation test for goodness of fit was applied to determine whether the observed samples were normally distributed or not. If the parameters were normally distributed, treatments were compared by applying one-way ANOVA. Post hoc test (LSD) was performed. If the parameters were non-parametric, treatments were compared by applying the Mann-Whitney two samples (non-matched) test. The difference was considered significant at ($P \leq 0.05$).

RESULTS

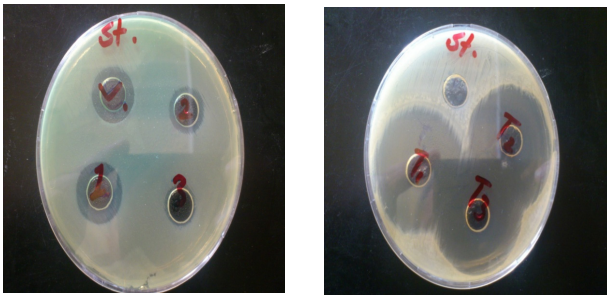
Penicillin G at concentrations of 30 mg.ml^{-1}and 60 mg.ml^{-1} was not found to have any inhibitory effect on the growth of *S. aureus* (Figure 1a, (3) and (2), respectively). It was clearly observed that both the control (PBS) (Figure 1b (V)) and Penicillin G at these concentrations displayed no zone of inhibition. However, an elevated dose of Penicillin G (120 mg) displayed a significant effect on *S. aureus* (Figure 1a (1)) represented by a clear ring of growth inhibition. This zone of growth inhibition was also observed for the treatment with Vitamin C alone at a concentration of 10 mg (Figure 1a (V)). Interestingly,

the zone of inhibition for the effective dose of Penicillin G (120 mg) was distinctively less than that for Vitamin C (10 mg), suggesting that Vitamin C alone acts as an effective antibacterial agent. Surprisingly, all treatments combining Vitamin C with each dose of Penicillin G displayed a clear zone of inhibition (Figure 1b) when compared to the control. This revealed a potentiating effect of Vitamin C on the effect of Penicillin G even at lower doses and the zones of inhibition of the combined treatment (Penicillin G and Vitamin C) were much greater than those of the Vitamin C and Penicillin G alone. As the extent of the zones of inhibition is greater in the combination treatments than for the sum of their singular treatments, it may be suggested that there is a synergistic effect between Vitamin C and Penicillin G. Replicate data show no significant variation (Table 1).

Table 1: The effect of Penicillin G on *S. aureus* in the presence of Vitamin C expressed as the zone of inhibition (mm).

Treatments	Zone of inhibition
Control	0.00 ± 0.00
Vitamin C (10 mg)	3.40 ±0.24 *
Penicillin G (120 mg)	2.20 ± 0.91 *,a
Penicillin G (60 mg)	0.20 ± 0.20 a
Penicillin G (30 mg)	0.00 ± 0.00 a
Penicillin G (120 mg) + Vitamin C	14.20 ± 0.20 *, a, b
Penicillin G (60 mg) + Vitamin C	13.60 ± 0.24 *, a, b
Penicillin G (30 mg) + Vitamin C	13.40 ± 0.40 *, a, b

* Significant difference compared to control at $P \leq 0.05$; **a**, significant difference compared to Vitamin C at $P \leq 0.05$; **b**, significant difference compared to the same dose of Penicillin G alone at $P \leq 0.05$.



(a) **(b)**

Figure 1: The effect of Penicillin G on *S. aureus* in the presence of Vitamin C 10 mg.
(a): Vitamin C (V), Penicillin G 120 mg (1), Penicillin G 60 mg (2) and Penicillin G 30 mg (3).
(b): Penicillin G 120 mg + Vitamin C (T1), Penicillin G 60 mg + Vitamin C (T2), Penicillin G 30 mg + Vitamin C (T3) and control (C).

In the case of the Gram-negative bacterium *E. coli*, Penicillin G was found to be ineffective only at the lowest dose of 30 mg (Figure 2a (3)), seen by the absence of a zone of growth inhibition. However, Penicillin G at doses of 60 mg and 120 mg displayed a significant inhibition on the growth of *E. coli* (Figure 2a (3) and (2), respectively) as compared to the control (Figure 2b (C)). As with *S. aureus*, Vitamin C alone was found to inhibit the growth of *E. coli* (Figure 2a (V)). However, the zone of inhibition was found to be similar, but not greater, to the extent of inhibition when using Penicillin G alone at the highest concentration (Figure 2a (1)). Similarly, the treatment of *E. coli* with Vitamin C at a dose of 10 mg in combination with Penicillin G at all concentrations (including 30 mg), resulted in larger zones of inhibition than with Penicillin G alone (Figure 2b). The observation that Vitamin C potentiates the antibacterial effect of Penicillin G even at a lower, previously ineffective concentration (30 mg), (as the zone of inhibition observed for the combination of Vitamin C and 30 mg Penicillin G is greater than that with Vitamin C alone) is very interesting for potential medical applications. However, it is also important to note that, although there is a similar enhancement of the zone of inhibition by Penicillin G in the presence of Vitamin C in *E. coli*, there may be a different mechanism involved, as the increase was observed to be only additive. Therefore, Vitamin C may impose its effect in potentiating the antibacterial activity of Penicillin G differently in *E. coli* and *S. aureus*. Replicate data show no significant variation (Table 2).

Table 2: The effect of Penicillin G on *E. coli* in the presence of Vitamin C expressed as the zone of inhibition (mm).

Treatments	Zone of inhibition
Control	0.00 ± 0.00
Vitamin C (10 mg)	3.00 ± 0.31 *
Penicillin G (120 mg)	3.00 ± 0.00 *
Penicillin G (60 mg)	1.20 ± 0.48 *,a
Penicillin G (30 mg)	0.00 ± 0.00 a
Penicillin G (120 mg) + Vitamin C	5.20 ± 0.37 *, a, b
Penicillin G (60 mg) + Vitamin C	4.80 ± 0.37 *, a, b
Penicillin G (30 mg) + Vitamin C	4.40 ± 0.24 *, a, b

* Significant difference compared to control at $P \leq 0.05$; **a**, significant difference compared to Vitamin C at $P \leq 0.05$; **b**, significant difference compared to the same dose of Penicillin G alone at $P \leq 0.05$.

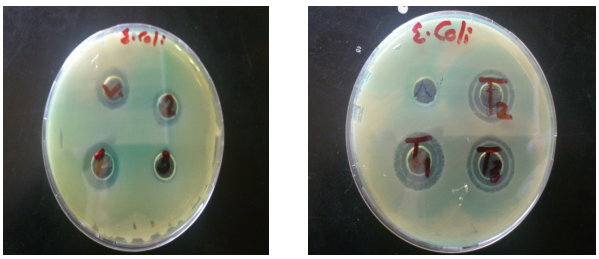


Figure 2: The effect of Penicillin G on *E. coli* in the presence of Vitamin C 10 mg.
(a): Vitamin C (V), Penicillin G 120 mg (1), Penicillin G 60 mg (2) and Penicillin G 30 mg (3).
(b): Penicillin G 120 mg + Vitamin C (T1), Penicillin G 60 mg + Vitamin C (T2), Penicillin G 30 mg + Vitamin C (T3) and control (C).

In this context, the susceptibility and the sensitivity of the *S. aureus* to the combined treatment of Penicillin G and Vitamin C was significantly ($P \leq 0.05$) higher than that of the *E. coli* (Figure 3).

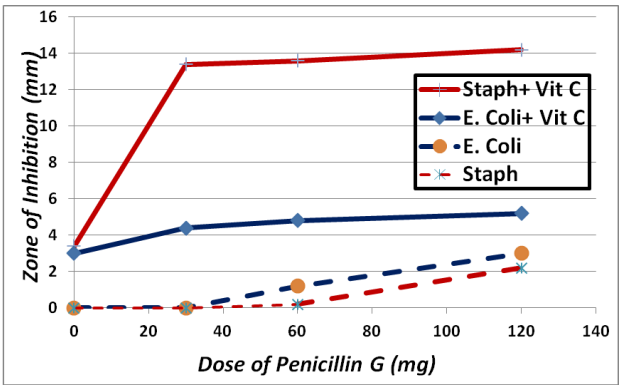


Figure 3: Graph shows the effect of Penicillin G on *S. aureus* and *E. coli* in the presence and absence of Vitamin C.

DISCUSSION

Since their introduction, the emergence of bacterial resistance to antibiotics has become increasingly evident for every major class of antibiotic, particularly the persistence displayed by *Neisseria gonorrheae* to cephalosporines.[31] It has previously been demonstrated that Vitamin C and some other vitamins are capable of increasing the permeability of Gram negative bacteria to antibiotics.[32,33] This is more specifically documented for the *P. aeruginosa* bacterium and given the name permeabilizers.[34] The property of ascorbate as a reducing compound (electron donor) may play an important role in its action on outer membranes.[34] It has been estimated that the rate of permeation of β-lactam antibiotics across the *P. aeruginosa* outer membrane is about 12-fold less than that across the *E. coli* outer membranes.[35,36] Future research on ascorbic acid-antimicrobial interactions may find new methods to control strains of resistant bacteria.

Studies conducted in the 1970's reported that mega doses of ascorbic acid in combination with antimicrobials inhibited the growth of *P. aeruginosa*.[37,38] It was found to exert a strong bactericidal action on Gram negative bacteria and it was concluded that ascorbic acid may act by competing with the magnesium binding sites in the cell wall, cell membrane or ribosomes.[37] It was suggested that ascorbic acid alters the cell surface to render it increasingly permeable to these antibiotics.[38] Other reports suggested that massive doses of ascorbic acid considerably broadened the activity spectrum of the antibiotics.[39,40] Similar studies showed that high doses of ascorbic acid, in combination with antibiotics, inhibited the growth of *Helicobacter pylori in-vitro*.[41-46] Other studies conducted on the susceptibility of Gram-positive bacteria displayed similar data. It has been suggested that ascorbic acid may induce the loss of R plasmids and affect the levels of antibiotic resistance in *Staphylococci*.[47-49] The enhancement of antibiotic activity or the reversal of antibiotic resistance by nonconventional antibiotics affords the classification of these compounds as modifiers, or helpers, of antibiotic activity.[50-53]

In this study, a high dose (120 mg) of Penicillin was required to produce a significant effect in inactivating *S. aureus,* whilst the lower doses of 30 mg and 60 mg showed no antibacterial activity. Such phenomenon of resistance could be, on the one hand, explained by the bacterium *S. aureus* being of the type that are penicillinase enzyme producers which break down Penicillin G to penicilloic acid and thus make it inactive.[54] However, this is probably not the case as the same bacteria had responded to a higher dose of the antibiotic. On the other hand, some responsive bacteria show some insusceptibility when exposed to sub-inhibitory concentrations of the antibiotic. Further suggestion postulates that bacterial growth rate may be involved in this phenomena, where the slowest growing clones show no response to antibiotics.[11] Meanwhile, in this study, *E. coli* responded to a lower concentration of Penicillin G (60 mg) than *S. aureus*; such variable susceptibility could underlie the physiological differences between both bacterial species.

The combined application of Vitamin C with each dose of Penicillin G produced a significant increase in the activity of the antibiotic against both *S. aureus* and *E. coli*, compared to each dose of Penicillin G alone. This supports previous observations that state that Vitamin C potentiates the effect of Penicillin G. This may be mediated through altering the permeability of the bacterial cell surface leading to an increase in its permeability to Penicillin G[38] and other molecules. *S. aureus* was found to be more sensitive to the inhibitory effect of the combined treatment of Penicillin G with Vitamin C than *E. coli*. This may be due to the difference in the structure of the cell wall. Gram positive bacteria contain a penicillin binding protein which binds with Penicillin G and interrupts the process of peptidoglycan biosynthesis, this consequently leads to the bacterial cell wall being easily ruptured and deformed that facilitates bacterial death. Vitamin C was observed to potentiate this process, even with resistant *S. aureus*. Gram negative bacteria have an outer membrane, other than the peptidoglycan cell wall, which retards the access of the antibiotic to its target site. This means that Penicillin G needs to penetrate the outer membrane to reach the cell wall and give its effect.[54] Large doses of Vitamin C were shown to enhance the activity of Penicillin G against many resistant bacteria, as well as reduce allergic reactions to antibiotics.[43] Vitamin C thus, in effect, helps decrease the antibiotic resistance of bacteria and broadens the activity of antibiotics, to which bacteria are commonly resistant. The use of Vitamin C may also decrease the amount of antibiotics needed to kill bacteria.[40]

CONCLUSION

This work has demonstrated that Vitamin C itself exhibits an inactivating effect on the growth of both *E. coli* and *S. aureus*. This was clearly observed by the zone of inhibition of growth of the bacteria. When Vitamin C was used in combination with Penicillin G to challenge the bacteria,

S. aureus was found to be more susceptible than *E. coli*. Nevertheless, such combined therapy had a significant inhibitory effect on both bacterial species at various Penicillin G concentrations. Response was varied within the challenged bacteria as far as the doses deployed were within the sub-inhibitory (sub-effective) concentration. The outcome of this study could simply conclude that Vitamin C showed both a significant antibacterial effect on both Gram-positive and Gram-negative bacteria and exhibited a property where it enhanced the effect of the Penicillin G.

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