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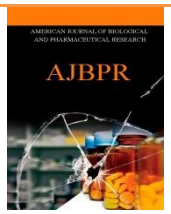

EVALUATION OF ULTRASTRUTURAL CHANGES INDUCED BY OFLOXACIN ASSOCIATED WITH CEPHALEXIN AGAINST HUMAN AND BOVINE STRAINS OF *STAPHYLOCOCCUS AUREUS* DURING POST ANTIBIOTIC EFFECT (PAE)

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ABSTRACT

The post-antibiotic effect (PAE) of ofloxacin alone and in association with cephalexin were determined in human strains of *Staphyococcus aureus* from a hospital and in bovine strains. Ofloxacin produced a persistent post-antibiotic effect (PAE) on all strains tested. The association of ofloxacin - cephalexin in strains of *S. aureus* generally produced an additive PAE. Ultrastructural changes such as intracellular electron-aggregates, changes in cytoplasmic texture and variations in cytoplasmic volume and cell shape were observed during the PAE using transmission electron microscopy. Cytochemical cell labeling for carbohydrate acids using ruthenium red by transmission electron microscopy showed differences in cytochemical staining between human and bovine strains during the PAE. The ultrastructural changes observed support the suggestion that one possible mechanism of PAE may be related to the persistence of the drug in connection sites, or alternatively of the time that the organism needs to synthesize new proteins and enzymes.

INTRODUCTION

The suppression of bacterial growth or post-antibiotic effect (PAE) followed by brief exposure of microorganisms to antimicrobial agents has been observed for decades. PAE is a pharmacodynamic parameter which provides information concerning the interaction between a antimicrobial agent and a microorganism and exerts an influence on the potential conduct of antibiotics [1, 2].

Fluoroquinolones, in particular ofloxacin, have a broad antimicrobial spectrum of antibiotic activity against gram-positive and gram-negative strains. The main action mechanism of fluoroquinolones is to inhibit DNA gyrase activity resulting in disruption of DNA synthesis, and consequently in bacterial cell death [3,4], in addition, quinolones are potent inducers of the SOS system of response, leading to filamentation in gram-negative bacilli [5,6]. These antimicrobial agents demonstrate persistent PAE, usually after 1 to 2 hours on a wide variety of microorganisms [7]. In vitro PAE has been reported for most antimicrobial agents on a wide variety of microorganisms. Numerous PAE studies have reported *Staphylococcus aureus* in clinical samples [8,9], although there are few reports on *S.aureus* in animal_samples. Studies have demonstrated PAE on several antibacterial strains of *S.aureus* causing bovine mastitis [10] .

The precise mechanism by which antimicrobials induce suppression of post-antibiotic growth has not been

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fully elucidated [11,12]. This persistent effect possibly involves multiple mechanisms and metabolic pathways, depending on the class of antibiotic and the microorganism [2,13]. Ultrastructural analysis is an essential parameter in order to understand phenomena that result in changes in metabolic behavior when conducting physiological and genetic studies of numerous cell types. Such studies seek to demonstrate ultrastructural changes in bacteria after exposure to antibiotics [14,15].

However, few studies report on the effects on ultrastructural changes by using cytochemical electron microscopy during the PAE phase, although such studies can be of great importance in understanding the nature of this phenomenon [13].

The purpose of this study was to determine the effect of post-index antibiotic ofloxacin alone and in combination with cephalexin on samples of human *S. aureus* from a hospital and bovine *S. aureus*. To futher clarify the antibiotic-microorganism phenomenon, ultrastructural analysis was conducted by transmission electron microscopy using the routine technique and acid carbohydrate cytochemistry to investigate the effect during the PAE.

MATERIALS AND METHODS

Bacterial samples

This study used two samples of animal *S. aureus* isolated from lactating bovines, and two samples of human origin, obtained from patients at the Lauro Wanderley University Hospital / UFPB (João Pessoa), which were characterized phenotypically as methicillin-sensitive and -resistant [16,17].

Inoculum of 10^6 CFU/ml

A 9 ml of bacterial culture was added to 1 ml of ofloxacin solution alone and in combination with cephalexin at a concentration four times greater than the MIC and controls were added to 1 ml tubes of sterile distilled water. After incubating for 2 hours at 37°C, the antibiotic activity was neutralized by a 1: 1 00, in Mueller-Hinton agar broth. The viable cell count in this tube and the time designated for the EPA were determined. Regrowth of culture was monitored every hour for a period of 6 hours, by the standard plate count method. The plates were read after incubation for 24 hours at 37°C. The experiments were performed in duplicate. Controls and the culture tubes containing the antibiotic concentration 1 : 100, were included in the experiment to ensure that the sub-inhibition of residual antibiotic does not affect the growth rate . The results were plotted on graphs, from which T and C (for interpolation) were calculated. The EPA content was calculated as per the equation: EPA = T - C where T is the time required for the cell count (cfu/ml) to be viable and the treated culture to increase by1 $\log_{10}$ count on being observed immediately after dilution. C is the time required for the control culture to increase by 1 $\log_{10}$ in the same conditions.

Ultrastructural study

Ultrastructural analysis was performed using the routine technique proposed by De Souza [18]. Cultures were exposed to ofloxacin isolates and association and aliquots were removed at 0, 2, 3 and 6 hours after removing the drug. A suspension culture, controls and the treated culture, was washed in phosphate buffered saline, pH 7.0, and centrifuged three times at 2000 rpm for 15 minutes. The supernatant was removed, and the cells were fixed in a solution of 2.5 glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2 for two hours at room temperature (25° C). Then the samples were washed in 0.1 M cacodylate buffer, pH 7.2. For post-mounting, we used osmium tetroxide 1 in 0.1 M cacodylate buffer, pH 7.2.

The organisms were dehydrated in solutions of increasing concentrations of acetone and embedded in Epon resin at 60° C for 72 hours. The blocks were cut with ultramicrotome and deposited on 300 mesh grids. The samples were observed and photographed with a transmission electron microscope (JEOL 100 CX).

Cytochemistry tests on acid carbohydrates was performed following the method proposed by Luft [19] to A 0.5 mg/ ml concentration of Red Ruthenium was added to the buffers used in the steps of fixing, post-fixation and washing as described above.

RESULTS

The results in terms of the MIC values for ofloxacin and cephalexin are shown in (Table 1). The duration of the post-antibiotic effect of ofloxacin and cephalexin for four strains of *S. aureus* exposed for 2 hours on a 4 x MIC concentration is shown in (Table 1). The ultrastructural study using the method of routine Transmission electron microscopy showed that, immediately after removing ofloxacin, all cyto-plasma samples exhibited the presence of granular space and increased, translucent cytoplasmic electrondensity. The results in terms of the MIC values fore ofloxacin and cephalexin are shown in (Table 1). The duration of the post-antibiotic effect of ofloxacin and cephalexin for four strains of S. aureus exposed for 2 hours on a 4 x MIC concentration is shown in (Table 1). The ultrastructural study using the routine transmission electron microscopy method showed results in the figures 1 and for human strains and 3 and 4 for bovine strains of *Staphylococcus aureus.* The results sowed that immediately after removing ofloxacin, all cyto-plasma samples exhibited the presence of granular space and an increase in translucent cytoplasmic cell electrondensity. A large number of cells had an increase in their volume

compared to a control sample not treated with antibiotics, not observing cross-wall of *Staphyloccocus aureus*, as shown in (Figures 1a and 1b).

After 3 hours of regrowth, "cell ghosts" were observed, indicating the death of the bacterial population. The lineage of human *S. aureus* from the hospital showed thicker electron dense granules on the outer surface of the cell wall, but this was not observed in the bovine sample, as shown in the (Figures 3a). After 6 hours of regrowth, decrease in the number of cell "ghosts" was observed.

In (Figure 2) TEM micrographs are shown of the human sample treated with ofloxacin and cephalexin. Structural variations similar to ofloxacin were observed. (Figure 4) shows SEM micrographs of the bovine sample treated with cephalexin and ofloxacin, respectively. Similar changes in the sample human were observed. However, the presence of electron-dense granules on the cell surface of the bovine sample is clearly visible.

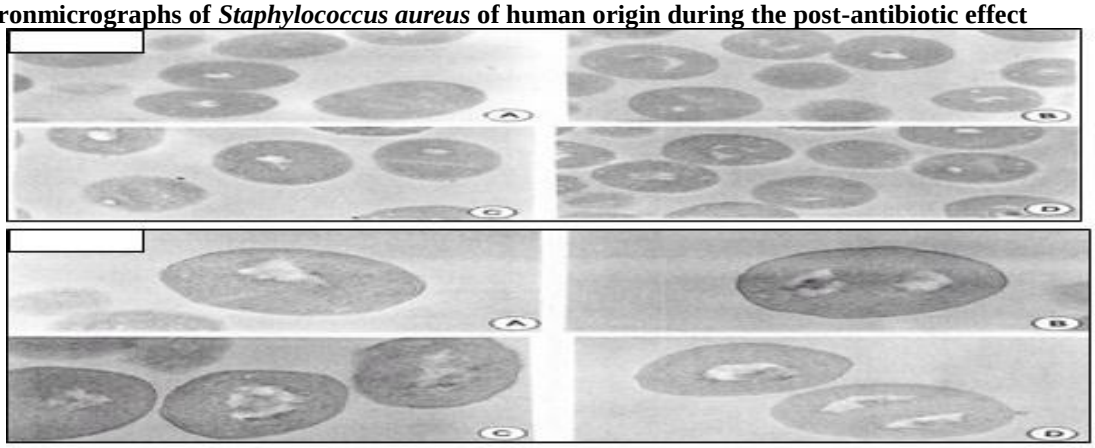
Cytochemistry tests on acid carbohydrates, by using ruthenium red in transmission electron microscopy, permitted a cytochemical reaction to be produced on the surface of the external cell wall in the form of granular and fibrillar material in all samples.

Table 1. Minimum inhibitory concentration (MIC) and post-antibiotic effect (PAE) ofloxacin and cephalexin in strains of *Staphylococcus aureus* isolated from human hospital and bovine strains

Strains	Antibiotic	CMI (µg/ml)	EPA (h)
Staphylococcus aureus/ Human strains	Cephalexin	2	0.72
01H	Ofloxacin	1	2.92
	Ofloxacin+		
	Cephalexin		3.6
	Cephalexin	2	0.48
*189C	Ofloxacin	2	1.44
	Ofloxacin		
	Cephalexin		1.28
Staphylococcus aureus/ Bovine strains	Cephalexin	2	0.68
	Ofloxacin	1	1.28
311FN	Ofloxacin		
	Cephalexin		2.2
	Cephalexin	2	0.84
	Ofloxacin	1	1.64
319U	Ofloxacin		
	Cephalexin		2.76

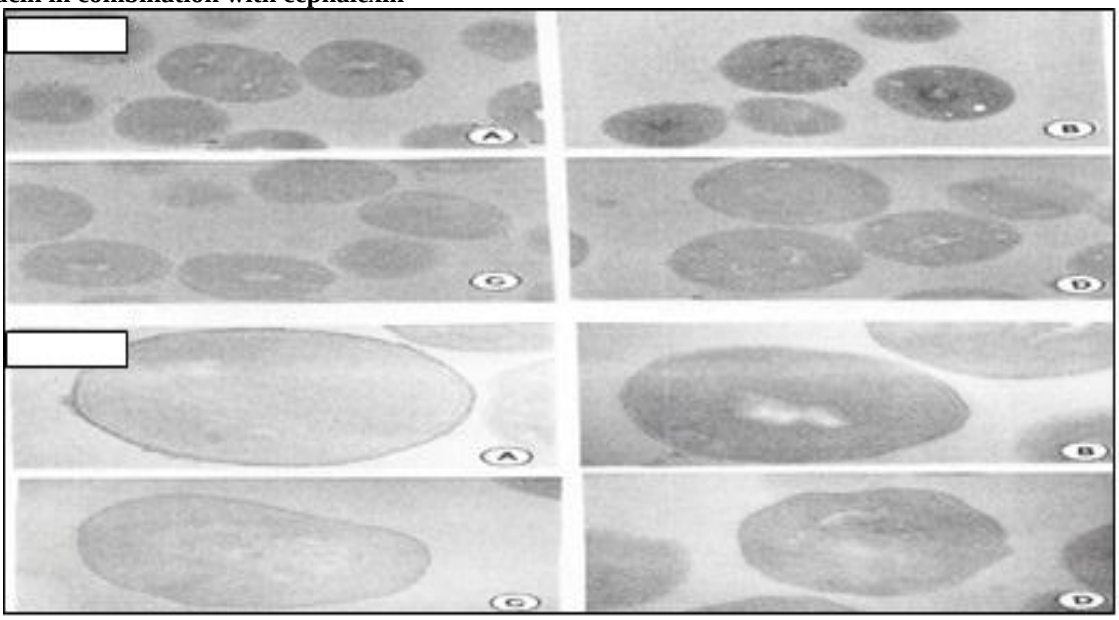
* *Staphy/ococcus aureus* Methicillin-resistant (MRSA)

Fig 1. Electronmicrographs of *Staphylococcus aureus* of human origin during the post-antibiotic effect



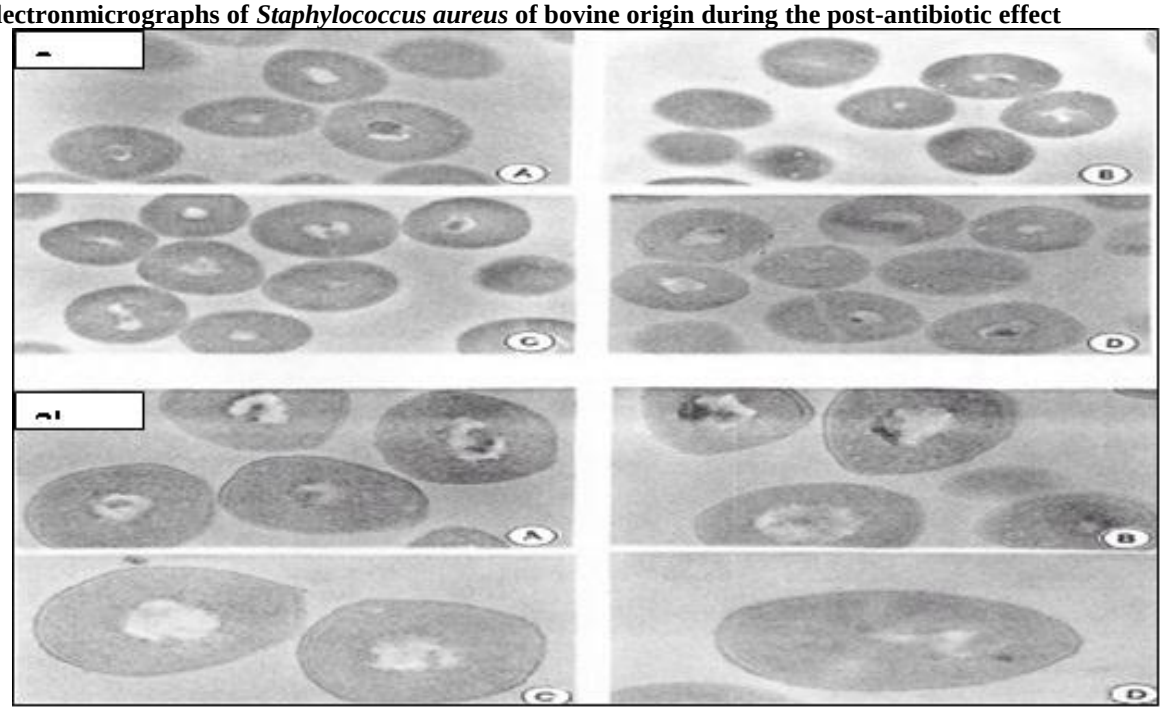
1a- Routine procedure - Treated with ofloxacin A - 0h X.80, B - 2h X.80, C X 3h X80, 6h X.100, and 64h of regrowth
1b- Cytochemical acids to carbohydrate ruthenium red. Sample treated with ofloxacin. A - 0h x.ao, B - 2h x80, C -3h X.80, D-6h X80, and 64h of regrowth.

Fig 2. Electronmicrographs of *Staphylococcus aureus* of human origin during the post-antibiotic effect. Strains treated with ofloxacin in combination with cephalexin



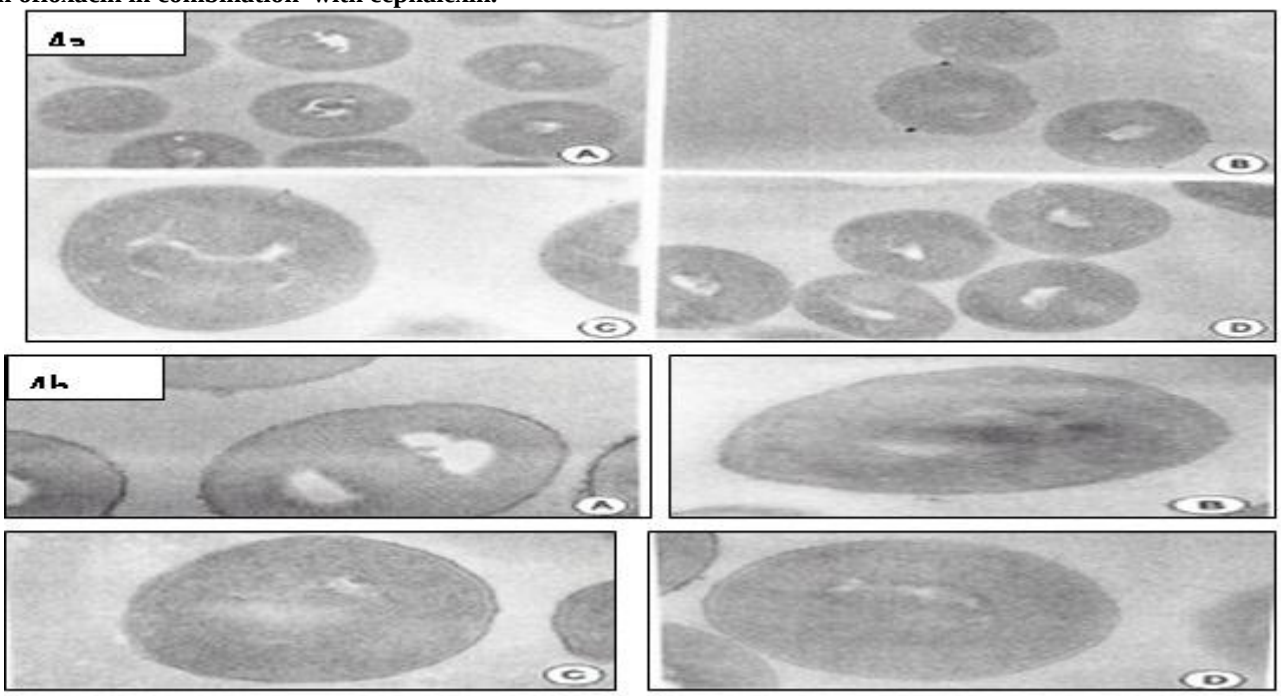
2a- Routine procedure - A - 0h X. 80, B - 2h X. 80, C - 3h X80, 6h X80, and . 64h of regrowth
2b- Cytochemical acids to carbohydrate ruthenium red. Sample treated with ofloxacin. A - 0h x.60, B - 2h x100, C -3h X.100, D-6h X100, and 64h of regrowth .

Fig 3. Electronmicrographs of *Staphylococcus aureus* of bovine origin during the post-antibiotic effect



3a- Routine procedure. Treated with ofloxacin A - 0h X. 80, B - 2h X. 80, C - 3h X80, D- 6h X80, and 64h of regrowth
3b- Cytochemical acids to carbohydrate ruthenium red. Sample treated with ofloxacin. A - 0h x.60, B - 2h x100, C -3h X.100, D-6h X.100, and 64h of regrowth.

Fig 4. Electronmicrographs of *Staphylococcus aureus* of bovine origin during the post-antibiotic effect. Strains treated with ofloxacin in combination with cephalexin.



3a- Routine procedure. A - 0h X. 80, B - 2h X. 80, C - X 3h 80 and 0 - 6h X. 80 and 64h of regrowth
3b- Cytochemical acids to carbohydrate ruthenium red. Sample treated with ofloxacin. A - 0h x.60, B - 2h x100, C -3h X.100 and D-6h X.100 and 64h of regrowth .

Differences in cytochemical staining were observed between human and bovine strains. The human sample showed higher intensity marking during the PAE than the sample of bovine origin (Figures 1b and 2b, and 3b and 4b).

DISCUSSION

In the last two decades, the number of studies that address the pharmacodynamics of antimicrobial agents has increased progressively. PAE is one of the most important and best-known pharmacodynamic parameters [20]. The results presented here indicate that both antibiotics, ofloxacin and cephalexin, induce a significant PAE on the strains of S. aureus tested, these data being consistent with the results obtained by other authors [8,9,21]. Fluoroquinolones produce a higher PAE on gram-positive counts than some f3 –lactams; cephalosporins produce a PAE between immediate and short duration (0,5 - 2h); while imipenems produce long term PAE [2]. In general, the results suggest that the influence of the association of ofloxacin with cephalexin was additive to PAE, but a synergistic effect was not observed. This additive effect may reflect the time required by the bacterium to synthesize new PBPs (penicillin binding proteins), or alternatively reflects the time required for dissociation to occur of the covalent interaction between β -lactams and the strains of *S. aureus* tested. These data are consistent with the results obtained by other authors [8,9,21].

Significant ultrastructural changes were observed in transmission electron microscopy, associated with the variation in electrondensity of the cytoplasmic cell texture in volume and the shape of the cells after treatment, do not observing "cross-walls. This heterogeneity in ultrastructural changes suggests that antibiotics affect organisms to different degrees, and are correlated with the duration of PAE which is determined by counting viable cells. At the end of the PAE, there was a reduction in the number of cell "ghosts" as well as the presence of electron-beads on the surface of cells, which may suggest the limited persistence of the drug binding site or a periplasmic space. Some cells showed a higher cell volume. However most of the cells showed a normal ultrastructure (Figures 1a and 3a).

Ultrastructural changes were observed in bacteria by Gottfredsson et al. [13], during the PAE, using transmission electron microscopy. Electron-aggregates were observed in strains of *S.aureus* exposed to dicloxacillin; these aggregates in *S. aureus* are associated with a decrease in the number of ribosomes, which had been previously shown in E. coli during continuous exposure to naked aminoglycosides. Other reports show that after exposure of *S. aureus*, ciprofloxacin showed an

increase in cell volume (0.8 to 1.2 in diameter) representing 70 – 80% persistence of the population during the PAE [13].

Ofloxacin in combination with cephalexin induced an additive PAE and produced ultrastructural changes similar to those observed with ofloxacin alone. Lorian et al [22] demonstrated morphological changes in *E. coli* after exposure to ampicillin and ciprofloxacin, resulting in the formation of morphological filamentation, arbitrarily setting the PAE time for bacterial population reverting to 80 in the form of shaped bacilli and 10 filaments. Quinolones induce filamentation in gram-negative bacilli, with decreased septum formation and cell division [23]. The PAE produced by these quinolones may represent the time required for the drug to dissociate itself from its receptors and diffuse out of the bacteria [12]. During the PAE induced by imipenem, however, most of the cells showed a normal ultrastructure (Figures 2 and 4).

During the APS induced by imipenem on *Pseudomonas aeruginosa*, a large number of globoide cells was observed, which probably represents the preferential binding of imipenem penicillin-binding proteins PBP2, which was also observed by [22], *Proteus mirabilis* during exposure to another PBP 2.

The variation in the expression of surface carbohydrates has been much studied with regard to the composition, content and distribution of these molecules on the cell surface. This is recognizably related to several factors such as maturation, differentiation and cell-cycle phase, as well as to responses to environmental changes. Additionally, surface carbohydrates are related to interaction processes and can act as cell receptors, in addition to their structural role [18,19,24-26]. Few studies have reported on the variation in content, distribution and the presence of carbohydrates on the surface of acid microorganism acids subjected to treatment with antibiotics. This article demonstrates by using cytochemistry a variation in these molecules in response to treatment with ofloxacin alone or in combination with cephalexin. Differences in cytochemical staining were observed between human and bovine strains, during the PAE. Dual-action quinolones may exist, but, at least for gram-positive bacteria, it has been difficult to find one that meets other criteria for clinical usefulness [27]. Antibiotics with different mechanisms of action may vary with respect to their effects on the release and activity of immunostimulatory gram-positive bacterial components of the cell wall. Mashino et al. [28] observed a large increase in the release of lipoteichoic acid and peptideoglican induced beta -lactam antibiotics. Like antibiotics, protein synthesis inhibitors do not affect the release and peptideoglican synthesis inhibits the release of lipoteichoic acid. The ultrastructural changes observed support the suggestion that one of the possible mechanisms of PAE may be related to the persistence of drug binding sites, or alternatively, may represent this as long as the body needs to synthesize new proteins and enzymes, culminating with the return to metabolic activities. Studies associated with other types of molecular variation should be conducted so that the PAE phenomenon may be better understood.

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