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Morphology Changes and Inhibition of Enzymes Production During PAE Induced by Clindamycin and Chlorhexidine Against Oral Streptococci

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Abstract: The study was conducted to determine the post antibiotic effect (PAE) of clindamycin and/or chlorhexidine against clinical isolates of oral streptococci. The PAE period was determined by spectrophotometric technique. A prolonged period of PAE (4h) was demonstrated when the isolate of *Streptococcus mutans* was exposed for an hour at a concentration of 4XMIC of clindamycin. While exposure to clindamycin at sub-MIC (1/4 and 1/8 XMIC) induced a short period (0.8 & 1h) under the same conditions. However, a short period (0.2 h) was obtained when the other isolate of *Streptococcus mutans* exposed to chlorhexidine at supra-MIC (8XMIC) for an hour under the same conditions. The impact of PAE on cell morphology and physiology during PAE was demonstrated. Thus, the morphology of streptococci was changed during PAE induced by ½ and 2XMIC of clindamycin. A marked reduction in cell surface hydrophobicity was observed upon exposure of the cells to clindamycin and chlorhexidine at 2XMIC for an hour. A significant decrease in elastase production by *Streptococcus mutans* was observed during PAE when the cells were exposed to 4XMIC of chlorhexidine. The hemolysin activity was significantly reduced by 30% during PAE induced by exposure of the cells to 2XMIC of both clindamycin and chlorhexidine for an hour.

Key words: Post antibiotic effect · Streptococci · Clindamycin · Chlorhexidine · Cell surface hydrophobicity · Elastase · Hemolysin production

INTRODUCTION

It is well known that the period following exposure of the bacteria to antibiotic for a specific period as the post antibiotic effect (PAE). PAE studies have been conducted with many antibiotic-organism combinations and most of these studies show some evidence of a PAE. It has been suggested that the PAE should be considered in designing antibiotic dosing regiments [1]. Clindamycin is usually recommended in the treatment of gingivitis and other oral diseases with aerobic and anaerobic infection. Clindamycin has demonstrated a prolonged PAE against different bacterial species [2]. Chlorhexidine is also used locally as a mouthwash for treatment of oral infections. In most case, combination of clindamycin (systematic) and chlorhexidine (topical) are used for treatment of oral infections. The purpose of this study was to determine the PAE of clindamycin and/or chlorhexidine against oral streptococci and the impact of this period on the physiology and the ultrastructure of the oral streptococci.

MATERIALS AND METHODS

Antimicrobial Agent: Clindamycin (sigma Aldrich, St. Louis, Mo) was used to prepare a stock solution of (150 µg/ml) in sterile distilled water. Clorhexidine hydrochloride sterile powder (ATA technologies limited) was dissolved in sterile distilled water to prepare a stock solution of (100 µg/ml), stored at -20°C. The stock was stable for 6 months. The antibiotic and antiseptic were suspended/diluted in Todd Hewitt Broth (THB) (LAB M™) during the exposure period (1 h) of the streptococci were sub-cultured onto blood agar and incubated overnight. The following day, a logarithmic-phase culture was obtained by inoculating two colonies into (THB) and incubating them in a shaking water bath at 37°C until the optical density was the same as a 0.5 McFarland standard.

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Fig. 1: Growth of *Streptococcus mutans* on Mitis Salivarius Agar (blue gum drop shape)

Bacterial Culture: Sreptococcal isolates were recovered from clinical specimens of patients suffered from gingivitis and/or oral microbial disease such as caries and plaques. Growth on Mitis-Salivarius agar was used also for characterization of the streptococcal isolates, the colonies showed blue gum drop shape which very characteristic for *Streptococcus mutans* [3] (Fig. 1). A single colony of each isolates was picked and subculture on Mitis-Salivarius agar slants. Plates were incubated aerobically at 37°C for 24 to 48 h and then stored at -4°C with 50% glycerol to maintain them for long–term storage about 3 months [4]. The isolates were identified to the genus level by the conventional microbiological techniques. API system was applied to all of the isolates as being the most helpful, rapid and reliable identification system for complete identification of isolates as *Streptococcus mutans*.

Determination of Minimum Inhibitory Concentration (MIC): The MIC values for the two isolates of the oral streptococci with the two drugs were determined by the agar dilution technique according to the procedures recommended by CLSI 2007 [5]. The MIC was determined visually, following 24h of incubation at 37°C. The MIC is defined as the lowest concentration of the drug that inhibited growth of bacterial cells, as indicated by the absence of turbidity (optically clear). Also, the MIC value for the two isolates of the oral streptococci with the two drugs were determined by the agar diffusion technique BSAC [6] by performing ten-fold dilution of the drug in Petri dish using aninoculumns of $2x10^5$ cfu/ml. The MIC was determined visually following 24h of incubation at 37°C. As indicated by absence of growth in the plates that inoculated with 20 µl on the surface of the blood agar plates. All experiments were repeated on two isolates occasions with triplicate determination on each occasion.

Preparation of Bacterial Suspension for PAE Determination: Streptococci cells, maintained a Mitis-Salivarius agar, were inoculated onto fresh slants and incubated overnight at 37°C for 24 h prior to use. The organisms were harvested and cell suspension prepared in sterile saline (0.9 %) NaCl and adjusted at optical density to equivalent 0.5 McFarland standard. Cultures were added to flasks containing Todd Hewitt Broth to give an initial density of 10^6cfu/ml. The flasks were then incubated at 37°C for an hour on a rotary incubator working at 190 rpm. Following this limited exposure, the drugs were removed by two cycle of dilution with broth and saline, respectively. Samples had been centrifuged for 15 minutes at 6000 rpm. Afterwards, the supernatant was completely decanted and the cell pellets were re-suspended in Todd Hewitt Broth followed by viable counts of the control to determine the initial cell density (at zero time).

PAE Assay: PAE determination was carried out according to the method described by Ramadan *et al.* [7]. The inocula were prepared by incubating the bacteria overnight at 37°C in Todd Hewitt Broth. The culture was then diluted 1:25 in fresh Todd Hewitt Broth and incubated in an incubator shaker until the logarithmic phase of growth was reached. Cultures at a cell density about 5 X 10^6cfu/ml were then exposed to various drug concentrations for an hour. Growth of the bacterial cells was monitored each hour to evaluate change in the turbidity by the measuring the optical density at 550 nm for a period of 24 h. The duration of PAE was calculated by using the formula PAE= T–C, where T was the time required for the relative optical density of the drug-exposed cell suspension to reach the 0.05 absorbance level after removal of the drug and C was the time required for the relative optical density of the drug-free control cell suspension to reach the same absorbance level. Thus T-C expressed the time in which the antibacterial agent was capable of causing growth suppression of the organism following limited exposure to the drug.

Scanning Electron Microscopy: Transmission electron microscopy was used to examine the ultrastructure changes in bacterial cells during the PAE period. The PAE was induced by exposing a fresh culture in the logarithmic phase to clindamycin and/or chlorhexidine at different concentrations. The culture was incubated in an incubator shaker for an hour at 37°C. A sample from the exposed culture was taken after an hour of drug removal (within PAE period). Bacterial films were prepared from

both treated and untreated cultures. These films were stained for 60 minutes with uranyl acetate, followed by 5 minutes with lead citrate [8]. The bacterial cells were examined with a Jeol JEM-100S transmission electron microscope (Jeol, Japan) operated at 80 KV accelerated voltage. Final magnification was 10000-25000X.

Cell Surface Hydrophobicity (CSH): Cell Surface Hydrophobicity was determined during the PAE period according to the method described by Farley [9]. A MHB and Todd-Hewitt cultures in the logarithmic phase was exposed to the antibiotic for an hour at 37°C in the incubator shaker. Clindamycin and chlorhexidine were used at their respective concentrations. Residual antibiotic was removed by centrifugation at 6000 rpm for 15 minutes. The recovered cells were washed in PBS followed by centrifugation at 6000 rpm for 15 minutes. Treated bacterial cells were re-suspended in saline to a visual optical density equal to that of non-treated control culture. The culture was then centrifuged at 6000 rpm for 15 minutes and sediment cells were re-suspended in PUM buffer (pH 6.9) to 5 ml total volume of cell suspension in PUM buffer. One ml volume of ŋ-hexane was added and the mixture vigorously agitated during 2 minutes and left on the bench for 10 minutes at room temperature. The aqueous phase was removed from the bottom of the tube. The absorbance of bacterial suspension remaining in the aqueous phases was measured at 550 nm in a Shimadzu UV-160A Spectrophotometer (Japan). The indexes of the cell surfaces after exposure to clindamycin and/or chlorhexidine were determined according to techniques described by Garcia *et al*. [10] and Teixeira *et al*. [11]. Briefly, washed cells were added to η-hexane (1 ml), vigorously agitated during two minutes and left on the bench for 20 minutes at room temperature. The aqueous phase was removed from the bottom of the tube and its absorbency also measured (A 550 test) against phosphate buffer saline. The Hydrophobicity Index (HI) was calculated as follows: HI= (A550 control–A550 test)/A550 control. The values of HI indexes were indicated the ability of adherence to the epithelial cell by relation between the HI and ability of adherence.

Elastase Production: Culture in the logarithmic phase was exposed to a clindamycin or chlorhexidine at different concentrations for an hour at 37°C in an incubator shaker. Residual antibiotic was then removed by centrifugation at 6000 rpm for 15 minutes. The recovered cells were washed once with saline followed by centrifugation at 6000 rpm for 15 minutes. Treated bacterial cells were re-suspended in saline to a visual optical density equal to that of non-treated culture. A volume of both treated and non-treated cultures was transferred into pre-warmed broth and then incubated at 37°C for 60 minutes for cultures treated with antibiotics at different concentration. Elastase production was then determined according to the method of Grimwood *et al.* [12]. The elastase enzyme production was determined by measuring the absorbance of the supernatant at 400 nm.

Haemolysin Production: Haemolysin production was measured as described by Springer and Goebel [13] with the modifications described as follows: accumulation of hemolysin: one ml samples of bacterial cells were taken at intervals as described for the measurement of PAE. The cells were removed by centrifugation and the supernatant assayed for haemolytic production which was defined as (external haemolysin). The washed cell pellets were suspended into flask of 0.01 M PBS (pH 6) and sterile defibrinated beef blood. The flask was incubated at 37°C for an hour in incubator shaker at 190 rpm. After one hour, sample was taken to re-suspend into PBS (pH 6) and allow them to cenrifuge for 10 minutes. The cell debris was removed by centrifugation and the supernatant were tested for haemolytic production and defined as (internal haemolysin). Haemolytic production was determined by the amount of haemoglobin released from washed erythrocytes. The amount of released haemoglobin was measured by the absorbance of the supernatant at 495 nm.

RESULTS

MIC Determination: MICs of clindamycin and chlorhexidine were determined by agar dilution method. The results obtained showed that MICs for *Streptococcus mutans* isolates against clindamycin were 2, 16 and 1 µg/ml (Table 1), while MIC of the same isolates against chlorhexidine were 2.5, 5 and 10µg/ml. It is clear that the isolates 3 and 4 of *Streptococcus mutans* were sensitive to clindamysin while the isolate 1 and 2 were resistant, according to the criteria recommended by CLSI 2007 [5].

Relation Between MIC and PAE: A prolonged period of PAE (3 to 4 h) was obtained when the isolates of *Streptococcus mutans* (1 and 4) was exposed for an hour at concentrations of (16 and ½ XMIC) and (4, 8, 16 and ½ XMIC) of clindamycin, respectively. While exposure of isolate 2 to clindamycin at 2XMIC and isolate 3 to clindamycin at ¼ XMIC induced a short period (0.5 h and 0.8 h) respectively, under the same conditions (Table 2).

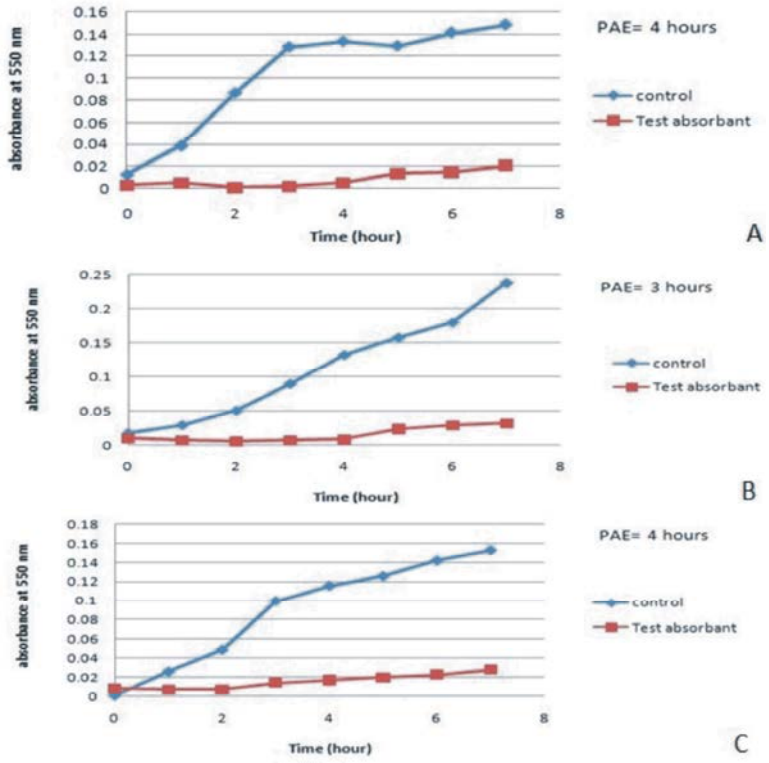
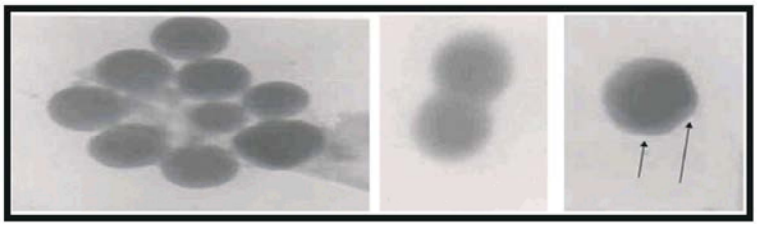
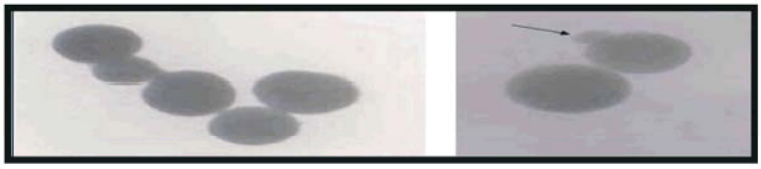


Fig. 2: PAEs induced by 1 hour exposure of *Streptococcus mutans 4* to clindamycin (A) 4XMIC; (B) 8XMIC and (C) ½ XMIC



A) *Streptococcus mutans* (control) 2XMIC clindamycin 1/2XMIC clindamycin



B) *Streptococcus mutans* (control) 2XMIC chlorhexidine

Fig. 3: Transmission electron microscopy of morphological changes induced by (A) 2XMIC and ½ XMIC clindamycin and (B) 2XMIC chlorhexidine against *Streptococcus mutans*, compared to untreated cells during PAE period

Furthermore, a longer period (5.5 h) was obtained when the isolate 4 of *Streptococcus mutans* exposed to chlorhexidine at supra-MIC (16XMIC) for an hour, while exposure of *Streptococcus mutans* isolate 4 to chlorhexidine at sub-MIC (1/16 XMIC) induced a short period of time (0.3 h) under the same conditions (Fig. 2).

Impact of PAE on Virulence Factors

Cell Morphology: *Streptococcus mutans* which exposed to (2 and ½ MIC) of clindamycin had morphological changes (by electron microscope) between them and their control, while exposing to (2XMIC) of chlorhexidine had a significant changes compare with the control (no-treated cells).

Table 1: MICs of *Streptococcus mutans* isolates to clindamycin and chlorhexidine

	*MIC ((µg/ml) for:	
Isolates number	Clindamycin	Chlorhexidine
1	2.0	2.5
2	16.0	5.0
3	1.0	10.0
4	1.0	10.0

* Average reading of MICs (Experiment was repeated 2 or 3 times)

Table 2: PAE period of clindamycin and chlorhexidine against *Streptococcus mutans* isolates.

	*PAE (h) induced by clindamycin at							
	SUPRA-MIC				SUB-MIC			
Isolates number	2 X MIC	4 X MIC	8 X MIC	16 X MIC	1/2 X MIC	1/4 X MIC	1/8 X MIC	1/16 MIC
1	1.0	2.0	2.3	3.0	4.0	3.0	2.5	2.0
2	0.5	1.0	1.5	2.0	3.0	1.8	1.5	1.5
3	1.0	1.0	2.0	2.0	0.8	0.8	1.0	1.5
4	2.5	4.0	4.0	4.1	2.0	2.0	1.0	1.0
	*PAE (h) induced by clindamycin at							
	SUPRA-MIC				SUB-MIC			
Isolates number	2 X MIC	4 X MIC	8 X MIC	16 X MIC	1/2 X MIC	1/4 X MIC	1/8 X MIC	1/16 MIC
1	4.3	4.3	4.5	5.0	1.0	1.0	0.2	0.2
2	2.0	3.0	1.8	1.0	0.3	0.2	0.2	0.2
3	0.3	1.0	1.5	1.0	1.0	0.8	1.0	0.5
4	0.2	1.0	4.0	5.5	0.5	0.5	1.0	0.3

*PAE was determined by Spectrophotometric technique

Table 3: Effect of clindamycin on CSH of *Streptococcus mutans* during PAE period using I ml of η-hexane

	Cell Surface Hydrophobicity			
			Hydrophobicity Index (HI) =	
Isolates number	A_{550} of aqueous phase of non-treated culture	A_{550} of aqueous phase of treated culture	(A_{550}control-A_{550}test)/A_{550}control.	
2	*0.065	2XMIC	1/2XMIC	2XMIC	1/2XMIC
		*0.035	0.027	0.584	0.585
4	0.222	2XMIC	1/2XMIC	2XMIC	1/2XMIC
		0.025	0.085	0.887	0.617

*Considered extremely significant P value is < 0.0001

Table 4: Effect of 2XMIC chlorhexidine on CSH of *Streptococcus mutans* during PAE period using I ml ç-hexane.

	Cell Surface Hydrophobicity		
			Hydrophobicity Index (HI) =
Isolates number	A_{550} of aqueous phase of non-treated culture	A_{550} of aqueous phase of treated culture	(A_{550}control-A_{550}test)/A_{550} control.
2	*0.026	2XMIC	2XMIC
		*0.014	0.462
4	0.004	2XMIC	2XMIC
		0.025	-5.250

*Considered extremely significant P value is < 0.0001

Table 5: Effect of combination of clindamycin/chlorhexidine at a concentration of 2XMIC on CSH of *Streptococcus mutans* during PAE period using I ml η-hexane

	Cell Surface Hydrophobicity		
Isolates number	A_{550} of aqueous phase of non-treated culture	A_{550} of aqueous phase of treated culture	Hydrophobicity Index (HI) = (A_{550}control-A_{550} test)/A_{550} control.
2	0.006	2XMIC	2XMIC
		*0.054	-0.048
4	0.004	2XMIC	2XMIC
		0.004	1.000

*Considered extremely significant P value is < 0.0001

Table 6: Elastase production by *Streptococcus mutans* during PAE induced by chlorhexidine at 4 and ¼ XMIC

Isolates number	Chlorhexidine concentration	Elastase production
*1	Control	0.033
	4XMIC	0.038
% of elastase production compared to control		86.80
**4	control	0.029
	1/4XMIC	0.035
% of elastase production compared to control	82.90	

*Considered very significant P value is 0.0088

**Considered very significant P value is 0.001

Table 7: Hemolysin production by *Streptococcus mutans* during PAE induced by clindamycin and chlorhexidine

Isolates number	Clindamycin concentration	Hemolysin production
2	Control	0.26
	2XMIC	0.24
	1/4XMIC	0.26
% of external hemolysin activity compared to control 2XMIC		92.30
% of external hemolysin activity compared to control 1/4XMIC		100.00
*4	Control	0.37
	2XMIC	0.31
	1/4XMIC	0.34
% of external hemolysin activity compared to control 2XMIC		83.78
% of external hemolysin activity compared to control 1/4XMIC		91.89
Isolates number	Clindamycin concentration	Hemolysin production
2	Control	0.26
	2XMIC	0.23
% of external hemolysin activity compared to control		88.46
*4	Control	0.37
	2XMIC	0.26
% of external hemolysin activity compared to control		70.27

*Considered very significant P value is 0.0018

**Considered extremely significant P value is 0.0002

Table 8: Hemolysin production by *Streptococcus mutans* during PAE induced by combination of both clindamycin and chlorhexidine at a concentration of 2XMIC

Isolates number	Clindamycin concentration	Hemolysin production
2	Control	0.26
	2XMIC	0.21
% of external hemolysin activity compared to control		80.77
*4	Control	0.37
	2XMIC	0.26
% of external hemolysin activity compared to control		70.27

*Considered extremely significant P value is 0.0002

Cell Surface Hydrophobicity: The effect of clindamycin and/or chlorhexidine on the CSH during the PAE period was studied. The antimicrobial agents were tested at 2XMIC and sub-MIC 1/2XMIC concentrations. The absorbance at 550 nm of the aqueous phase was measured after partitioning of the cells between aqueous and organic phases according to the method described by Ramadan [7] and Garcia [10]. A slight decrease in CSH was demonstrated by exposure of the *Streptococcus mutans* (2 and 4) to clindamycin more than chlorhexidine under the same conditions (Tables 3 and 4). The marked reduction in CSH was observed upon exposure of streptococcal isolates to combination of clindamycin and chlorhexidine (Table 5), under the same conditions.

Enzyme Production

Elastase Production: Elastase production was determined during PAE period by spectrophotometric technique at 400 nm of residual bovine neck ligament elastin according to the method described by Grimwood *et al.* [12]. The obtained data showed that chlorhexidine at concentrations of 4XMIC and 1/4XMIC significantly repressed elastase production during PAE compare to the control (Table 6).

Hemolysin Production: To compare the hempolysin activity between the antibiotic-free control and antibiotic treated cells, cell mass (e.g. 'per OD_{495}' (unit of optical density at 495 nm)) was used as a reference unit. Table 7 show the effect of 2XMIC and 1/4XMIC of clindamycin on external hemolysin activity in *Streptococcus mutans*. The % of external hemolysin activity of 2XMIC of clindamycin treated cells (*Streptococcus mutans* isolate 2) was about 92.30%, while at 1/4XMIC was 100%. Although, the % of external hemolysin activity of 2XMIC of clindamycin treated cells (*Streptococcus mutans* isolate 4) was decreased about 16.22%. On the other hand, the effects of chlorhexidine on external hemolysin activity in *Streptococcus mutans* were also obtained in Table 7 and hemolysin activity was significantly decreased about 29.73% such as the effect of 2XMIC of a combination of clindamycin and chlorhexidine on external hemolysin activity (Table 8).

DISCUSSION

Determination of the post-antibiotic effect is now an important part of preclinical evaluation of new antibiotics because it is a factor that influences antibiotic dosing intervals [14]. Prolongation of PAE period at highly concentration of drugs resulted from the impact of drug on bacterial cell featured. Clindamycin has been used clinically for more than 30 years and has demonstrated a good record of efficacy and safety in a variety of infections, including odontogenic infections [15]. The obtained data showed that the MIC of clindamycin against *Streptococcus mutans* isolates (1 and 2) were 2µg/ml and 16µg/ml, respectively. While MICs of the *Streptococcus mutans* isolates (3 and 4) were 1.0µg/ml. According to the criteria recommended by CLSI 2007 [5] four isolates were selected (two resistant isolates (1 and 2) and two sensitive isolates (3 and 4). The development resistance to clindamycin might be due to changes in outer or inner membranes and/or efflux pumps [16, 17]. Martinez *et al.* [2] and Ferrara *et al*. [18] reported that clindamycin showed higher activity against Streptococci than *Staphylococcus aureus*. Furthermore, Ciraj *et al.* [19] confirmed that the emergence of resistance to multiple antibiotics among gram-positive cocci has left very few therapeutic options for clinicians. Though 50% of their isolates were resistant phenotypes, the other 50% were sensitive to clindamycin, against which it would be safe and appropriate to use clindamycin or other macrolides. On the other hand, it is well known that chlorhexidine exerts its bactericidal effects by inducing alterations in the bacterial cell membranes [20]. Furthermore, some efficacy studies have demonstrated that chlorhexidine induces a more significant reduction of bacterial cultures than other antimicrobial compounds [21]. Therewith, the MIC values of chlorhexidine were in line with the observation of Dever *et al.* [22] who recorded increasing MICs of chlorhexidine for *Streptococcus mutans*. Chlorhexidine induced PAE (3-5 h) against *Streptococcus mutans* isolates (1 and 2) with MICs value of 2.5µg/ml and 5µg/ml, respectively. These results are in agreement with those obtained by Strijp *et al.* [23] who reported a significant reduction of *Streptococcus mutans* recovered from completely demineralized dentin treated with chlorhexidine. However, chlorhexidine sensitive isolates showed peeling of the outer membrane, a substantial loss of cytoplasmic electron-dense material and extensive lysis [24].

The data had been shown that clindamycin at supra-MIC (4XMIC) induced shorter PAE period (1 h) against *Streptococcus mutans* (isolate 2) than that induced against *Streptococcus mutans* (isolate 4) (4 h). On the other hand, clindamycin at sub-MIC (1/2XMIC) induced longer PAE period (4h) against *Streptococcus mutans* (isolate 1) than that induced at the same concentration against *Streptococcus mutans* (isolate 3)

(1 h). Thus, clindamycin at low concentrations (sub-MIC) prolonged PAE periods than high concentration (supra-MIC) against resistant *Streptococcus mutans*. These results are in agreement with that reported by Herbert and Richard [25] that PAE not always increase with increasing clindamycin concentrations. The concept of PAE should not only be considered as prolonged suppression of bacterial growth, but also as a potential inducer of decreased microbial virulence which may directly or indirectly influence the host parasite relationship [7]. The effect of antibiotics on the expression of streptococcal virulence factors in relation to the severity of infection and subsequent efficacy has been a topic of recent interest [26]. The impact of PAE induced by clindamycin and/or chlorhexidine on virulence factors of streptococci was examined. These factors are cell morphology, adherence, cell surface hydrophobicity, enzyme productions and hemolysin production. The data obtained that exposure of the cells in logarithmic phase to clindamycin and/or chlorhexidine at different concentrations induced a marked change in the cell morphology compared to the untreated cells which showed the normal spherical and cluster shape. Gottfredsson *et al.* [8] reported that during the PAE phase tobramycin induced ultrastructural changes against gram-positive cocci, such as a pattern of dense nuclear material and peripheral vacuoles. Moreover, the ultrastructure of MRSA strain showed undulating thick cell walls with mesosomes and thick cross walls of decreased electron density. These findings remained for at least 2h after arbekacin removal during PAE [7]. At an initial concentration of 2XMIC in a PAE test, macrolides were observed to induce an increase in *Staphylococcus aureus* size (up to two times larger) with an undulating outer layer and a thickened cross-wall linkage [27]. The ultrastructure of *Staphylococcus aureus* was changed during PAE induced by dicloxacillin [28]. Bacterial adherence is one of the major virulence factors of the oral streptococci. Virulent strains in some pathogens were more hydrophobic than non-virulent strains; therefore, hydrophobic characteristics of the bacterial surface are important in interaction between bacteria cells [29, 30, 31].

The hydrophobicity indexes of the bacterial surface of gram-positive after exposition to clindamycin were obtained during the PAE phase. The obtained results showed that exposure of the *Streptococcus mutans* to clindamycin at 2XMIC significantly reduce the cell surface hydrophobicity as same as chlorhexidine at the same concentration. With regard to great reduction in CSH was observed upon exposure of *Streptococcus mutans* to a combination of clindamycin and chlorhexidine at 2XMIC under the same conditions. Lorian and Gemmell [28] found that an adherence of treated bacteria is altered during the PAE phase. Bacterial adherence is influenced by the net surface charge and/or specific binding arrangement by host factors and by strain variation. It has been found that cell surface charge (hydrophobic/hydrophilic) of streptococci to hydrocarbon is altered after exposure to clindamycin [7, 32]. The decrease in hydrophobicity was unrelated to inhibition of growth and may be explained on the basis that clindamycin inhibits adhesion synthesis in streptococci [7]. Enzyme productions are one of the most virulence factors of streptococci. Chlorhexidine at a concentration of 4XMIC and 1/4XMIC repressed elastase production (13.5% and 17.5%) respectively compare to the control. Moreover, Clindamycin at concentrations of 1/2XMIC and 8XMIC against streptococcal isolates induced an increase in elastase production (106.5% and 96.9%), respectively. This finding was explanation by Shibl [33] who suggested that clindamycin at low concentration alters gram-positive cocci virulence properties apart from inhibiting growth. Nonetheless, combination of clindamycin and chlorhexidine at 2XMIC against *Streptococcus mutans* did not affect elastase production. Apparently, our finding is disagreement with that of Herbert [25] who reported the expression of protein A of *Staphylococcus aureus* is altered when the organism is exposed to this antibiotic at concentrations below the MIC leading to increase microbial susceptibility to phagocytosis and suggesting additional therapeutic efficacy.

Other studies suggest that the PAE may have an impact on toxin and bacterial enzyme production, which in turn may affect the host [35]. Some bacteria produce haemolysins, that are enzymes which cause the release of haemoglobin from erythrocytes and so they are considered as bacterial virulence factors [34]. The obtained data showed that the effects of 2XMIC of clindamycin on external hemolysin activity in streptococcal isolates. The percentage of external hemolysin activity of 2XMIC of clindamycin treated cells *Streptococcus mutans* was decreased about 16.22%. Furthermore, the percentage of external hemolysin activity of 2XMIC of chlorhexidine treated cells *Streptococcus mutans* was decreased about 29.73% such as effecting of combination of clindamycin and chlorhexidine at the same concentration against same isolates. In conclusion, a potentially significant prolongation of the PAE by combination of drugs was observed, but only if both (or all) agents induced a

PAE when used alone. The impact of this observation needs to be examined further in studies involving multiple and different dosing regimens in an infection model.

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