

Full Length Research Paper

Antibacterial effect of chlorine dioxide and hyaluronate on dental biofilm

F. Al-bayaty^{1,4,*}, T. Taiyeb-ali², M. A. Abdulla³ and F. Hashim³

¹Department of Restorative Dentistry, Faculty of Dentistry, University Teknologi MARA, Malaysia.

²Department of Oral Medicine and Periodontology, Faculty of Dentistry, University Malaya, Malaysia.

³Department of Molecular Medicine, Faculty of Medicine, University Malaya, Malaysia.

⁴Faculty of Dentistry, Level 19, Tower 2, Science and Technology Complex 40450, Shah Alam, Malaysia.

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The objective of this study is to investigate antimicrobial action of chlorine dioxide (ClO₂) gel and hyaluronate gel (Gengigel®) on dental biofilm. Pooled supra and subgingival dental biofilm were obtained from healthy individuals and incubated aerobically and anaerobically. Plaque bacteria investigated including *Streptococcus constellatus*, *Streptococcus mitis*, *Eikenella corrodens*, *Fusobacterium nucleatum*, dental plaque pool samples (aerobic and anaerobic) and *Staphylococcus aureus* and *Escherichia coli* as internal control microorganisms. All bacteria were grown in Brain Heart Infusion (BHI) broth and the clinical isolate strains were sub-cultured on BHI agar. Single pure colonies of bacteria were transferred into fresh BHI broth and incubated overnight. Bacterial counting was done using hemocytometer. Antibacterial activities were determined using bacteria grown on Mueller Hinton II agar and antimicrobial disc diffusion susceptibility testing with paper discs impregnated with ClO₂ and Hyaluronate gels as well as by minimum inhibition concentration (MIC) test. Bacterial morphological alterations following treatment with ClO₂ and Hyaluronate gels were viewed under Scanning Electron Microscope (SEM) at 3500x, 10000 x and 20000x magnification. Positive results were obtained with disc diffusion technique whereby both agents exhibited antibacterial action against the microorganisms tested. ClO₂ gel produced large diameter inhibition zones while Hyaluronate gel resulted in smaller diameter inhibition zones. In MIC test the lowest MIC value of ClO₂ gel (0.02% w/v) was obtained for *S. aureus*, *S. mitis* and *S. constellatus*. The other bacteria and pool samples of dental biofilm indicated slightly higher MIC values (0.2% w/v) for ClO₂ gel. However, MIC values for Hyaluronate gel could not be determined. Under SEM, ClO₂ gel produced obvious alterations to the bacterial morphology while no changes were observed after treatment with hyaluronate gel. Chlorine dioxide gel demonstrated stronger and obvious antibacterial activity.

Key words: Chlorine dioxide gel, Gengigel®, dental biofilm.

INTRODUCTION

Dental plaque is a complex biofilm formed on teeth surface composed of glucans and has the ability to resist antibiotics (Costerton et al., 1999). It is a thin layer of organic material consisting mainly dissolved food, mostly sugar and bacteria covering all or part of the tooth exposed surface (Jacob and Cate, 2006). The complex biofilm adheres firmly to the teeth surface which then

makes it so difficult to be removed if only by rinsing and water spray (March, 1994, 2003). A clinical study done by Loe et al. (1965) confirmed that plaque is the etiologic agent of gingivitis. There are over 500 bacterial species comprise plaque which makes plaque as the main etiologic agent of dental caries and periodontal disease (Rosan and Lamont, 2000).

Hyaluronic acid is composed of repeating disaccharides of D-glucuronic acid and N-acetyl-glucosamine which makes up a large nonsulfated glycosaminoglycan (Toole et al., 1989) present in gingival tissue (Giannobile et al., 1993). Gengigel® is an oral medicine product

*Corresponding author. E-mail: drfouadh@yaho.com. Tel: 00603-55435. Fax: 00603-55435803.

containing high molecular weight of hyaluronic acid, present as sodium hyaluronan (Pagnacco et al., 1997). It is specially formulated to resemble the hyaluronic acid found in normal gingival tissue with the addition of 2, 4-dichlorobenzene to enhance the antibacterial and anti-septic activity (Brandimarte, 1973; Fornara, 1992). The bacteriostatic effect of high molecular weight hyaluronic acid on various bacterial strains has been revealed by Pirnaza et al. (1999). A previous study reported that hyaluronic acid being able to reduce inflammation after dental surgery (Pagnacco et al., 1997). Due to the wide-ranging physiological activity of hyaluronic acid in the gingival tissues with a small number of published data concerned about the antibacterial effect of hyaluronic acid, the present study was initiated.

Chlorine Dioxide (ClO₂) is a small, volatile and highly energetic molecule derived from chlorine and being used in food processing industry, dental waterline treatment and also surface disinfection (Eddy et al., 2005). According to a study done by Eddy et al. (2005), ClO₂ is capable of completely killing *Enterobacter faecalis* within 30 min at higher concentration which then makes it as an effective endodontic irrigant. The efficacy of ClO₂ in treating periodontal disease owing to its antimicrobial action has been demonstrated by Spindler and Spindler (1998). Thus, considering the fact that ClO₂ has antibacterial effect, many companies have used ClO₂ as the main ingredient in the oral medicine, especially for treating periodontal disease. ClO₂ was selected for this study because of its strong antimicrobial properties, ease of use and widespread availability.

The main goals for this research were to investigate the antimicrobial action of Chlorine Dioxide (ClO₂) gel and Hyaluronate gel (Gengigel®) on dental biofilm and selected bacteria represent dental biofilm.

MATERIALS AND METHODS

Two different types of commercially available gels were used in this study. It included (Gengigel®) and ClO₂gel. Gengigel is manufactured by Ricefarma s.r.l, Germany Marketing Malaysia Sdn Bhd. It contains 0.2% w/w hyaluronic acid. ClO₂ gel was obtained from its manufacturer, (Puu Lih Biotechnology Sdn Bhd.) It has the concentration of 18% w/v.

Experimental microorganisms

Streptococcus mitis, *Streptococcus constellatus*, *Eikenella corrodens* and *Fusobacterium nucleatum* were selected to represent dental plaque bacteria and were obtained from Clinical Diagnostic Laboratory, University Malaya Medical Center. Pool sample of supragingival and subgingival dental biofilm taken from mouth with aid of sterilized Gracey curette No12 of medically healthy donor were tested aerobically and anaerobically. Standard strain of *Staphylococcus aureus* (ATCC 25923) and *Escherichia coli* (ATCC 25922) were used as internal control organisms throughout this study. The microorganisms were subcultured on Brain Heart Infusion (BHI) agar and also inoculated into BHI broth as stock cultures for use throughout the experiment later on.

Bacterial count

The microorganisms were counted using a hemacytometer to give an actual and precise number of organisms (1×10⁶ cells/ml) that were used through out the assessment of antibacterial activity. Methylene blue dye was used to differentiate viable cells from dead cells under light microscope prior to cell counting. Viable cells appeared bright color and ring shaped whereas dead cells were stained dark. Concentration of bacteria was calculated according to the following formula:

$$\text{Bacterial Conc. (cells/ml)} = (\text{Total viable cells counted in 4 squares}/4) \times \text{dilution factor} \times 10000$$

Assessment of antibacterial activity

Disk diffusion test

Screening of antibacterial activity was done by the disk diffusion test, which is normally used as a preliminary check to select gels with higher antibacterial activity (Christoflogiannis, 2001). Sterile paper disks were dipped into a sterile Eppendorf tube containing Gengigel®, ClO₂ gel and sterile distilled water (negative control). Then, the disks were dried out and kept in the cold room. The disk diffusion test was carried out on nonselective Mueller-Hinton (MH) agars. Sterile cotton bud was dipped into the broth containing the tested bacteria and the cotton bud was used to evenly streak onto the surface of MH agars. All the paper disks that have been inoculated with Gengigel® and ClO₂ gel were carefully placed onto the agar by using sterile forceps and the plates were incubated overnight in an inverted position. The diameter of inhibition zones were measured using a ruler to the nearest millimeter (mm) readings on the following day. The disk diffusion test was done in triplicate.

Minimum Inhibitory concentration (MIC) test

MIC test was performed to assess the antimicrobial efficacy of Gengigel® and ClO₂ gel. MIC test was carried out by diluting Gengigel® and ClO₂ gel using sterile distilled water into various concentrations, 18 - 0.0002% w/v for ClO₂ gel and 0.2 - 0.0002% w/w for Gengigel. Equal volume of broth containing bacteria suspension (0.5 ml of 1×10⁸ cells/ml) was added into each tube containing equal volume of Gengigel®, ClO₂ gel and sterile distilled water. MIC was determined after an overnight incubation at 37°C, by observing the turbidity of each tube. Confirmation test was done by streaking the tested solution using an inoculating loop onto Brain Heart Infusion (BHI) agar and was incubated overnight at 37°C. The MIC results were obtained based on this confirmation test. The lowest concentration of Gengigel® and ClO₂ gel managed to inhibit 99% of an overnight bacterial growth was regarded as the MIC value. Each MIC test was done in triplicate.

Assessment of bacteria morphology

Scanning electron microscope was used to evaluate the physical changes occur in the bacterial shapes and structures before and after treatment with Gengigel® and ClO₂ gel. The bacterial suspension was diluted using phosphate buffer saline (PBS) in a sterile Eppendorf tube. Then, the bacteria cells were adhered on nuclear pore and were fixed in 4% glutaraldehyde in 0.5 ml cacodylate buffer to preserve the bacteria shape and structure. Bacterial cells were further being processed and sputter coated with

Table 1. The average diameter of inhibition zone of various disks for the disk diffusion tests seen on the agar plates for each type of microorganisms tested (Diameter of disk used = 6 m).

Micro-organisms	Diameter of inhibition zone (mm)	
	Gengigel® (hyaluronic acid gel)	Chlorine dioxide gel
	0.2% w/w	18% w/v
<i>S. mitis</i>	9	45
<i>S. constellatus</i>	9	47
<i>E. corrodens</i>	8	43
<i>F. nucleatum</i>	7	40
<i>E. coli</i> (internal control)	8	42
<i>S. aureus</i> (internal control)	9	44
Dental biofilm pool sample (aerobic)	8	43
Dental biofilm pool sample (anaerobic)	7	41

gold prior to examination by SEM (Bogner et al., 2007).

RESULTS

The number of bacterial cells that was used throughout the whole experiment was 1×10^7 cells/ml. The disk diffusion test was done in triplicate. The average diameter of inhibition zones produced by each type of microorganisms tested were calculated and presented in the Table 1. Results showed, that Gengigel® produced very small diameter of inhibition zones, only a few millimeter larger than the original disks used. Meanwhile, ClO₂ gel produced large diameter of inhibition zones, ranging from 40 - 47 mm for all microorganisms tested including pool samples of dental biofilm.

The largest diameter of inhibition zones were seen for *S. mitis*, *S. constellatus* and *S. aureus*. These bacteria are classified into gram-positive cocci bacteria. It was the most susceptible to Gengigel® and ClO₂ gel. On the other hand, the least susceptible bacteria were *F. nucleatum* and pool sample of dental biofilm (anaerobic), they produced smallest diameter of inhibition zones for both agents compared to the other bacteria. In this study, gram -negative bacteria such as *E. coli* and *E. corrodens* were found to be moderately susceptible to Gengigel® and ClO₂ gel. Sterile distilled water used throughout this study as the negative control, does not produced inhibition zones.

ClO₂ gel was able to inhibit the growth of all experimental microorganisms at the concentrations 18 - 0.2% w/v. The most interesting finding was that gram- positive bacteria such as *S. mitis*, *S. constellatus* and *S. aureus* were more susceptible to ClO₂ gel compared to the other bacteria tested. The gram-positive bacterial growth was inhibited at 0.02% w/v ClO₂ gel concentration (Table 2). The lowest concentration of ClO₂ gel able to inhibit the growth of *E. coli*, *F. nucleatum*, *E. corrodens* and also pool samples of dental biofilm was 0.2% w/v.

Assessment of the bacterial morphology by examination under scanning electron microscope (SEM)

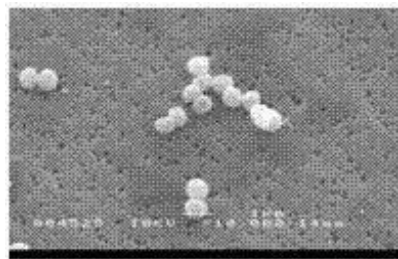
Dental biofilm bacteria were assessed for their morphological changes after treatment with Gengigel® and ClO₂ gel by (SEM). Four bacteria species were chosen to represent the periodontal health-associated bacteria, which were *S. mitis*, *S. constellatus*, *E. corrodens* and *F. nucleatum*. All of the bacteria were examined under general view of magnification (x3500) and specific view of magnifications (x10000 and x20000) to get better morphological details. SEM results before Gengigel® and ClO₂ gel treatment showed that all bacteria species were in normal shapes, *S. mitis* and *S. constellatus* were present in shape of cocci meanwhile *E. corrodens* was in shape of nice cocco- bacilli. Same goes with the scanning electron micrograph of *F. nucleatum* before undergoing treatment. A fusiform-shaped bacteria with smooth surface was observed.

These four bacterial samples were viewed by SEM after treatment with Gengigel® and ClO₂ gel to assess their morphological changes. No physical changes were observed after treatment with Gengigel®. The bacterial cells were still viable, shapes and structures remained the same as they were before treatment. However, obvious morphological changes seen on the bacterial shapes and structures after ClO₂ gel treatment. *S. mitis* shrank and formed irregular shape losing its cocci shape (Figure 1).

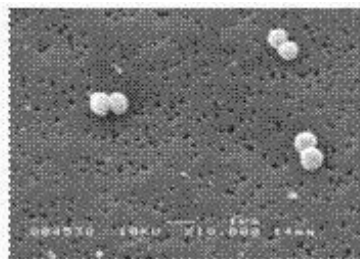
Similar result was obtained for *S. constellatus* in which the bacterial cells shrank after treatment with ClO₂ gel (Figure 2). The scanning electron micrograph of *E. corrodens* showed a slight different in structure compared to those Streptococcus group. The coccobacilli shape of *E. corrodens* was distorted. ClO₂ gel particularly on these bacteria causing rupture of bacterial cell wall, thus out bursting the cell contents (Figure 3). Viable bacterial cells maintain smooth cell surface while dead cells no longer

Table 2. The lowest concentrations of Gengigel® and chlorine dioxide gel able to inhibit the visible growth of the microorganisms after an overnight incubation.

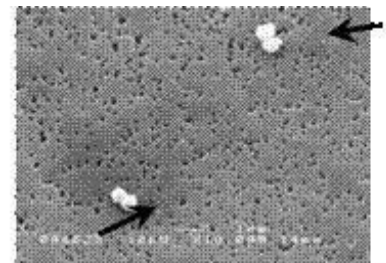
Micro-organisms	Mic value	
	Gengigel® (hyaluonic acid gel) (%w/w)	Chlorine dioxide gel (%w/v)
<i>S. mitis</i>	>0.20	0.02
<i>S. constellatus</i>	> 0.20	0.02
<i>Eikenella corrodens</i>	> 0.20	0.20
<i>Fusobacterium nucleatum</i>	> 0.20	0.20
<i>E. coli</i> (internal control)	> 0.20	0.20
<i>S. aureus</i> (internal control)	> 0.20	0.02
Dental biofilm pool sample (aerobic)	> 0.20	0.20
Dental biofilm pool sample (anaerobic)	>0.20	0.20



S. mitis before treatment with Gengigel® and Chlorine Dioxide gel

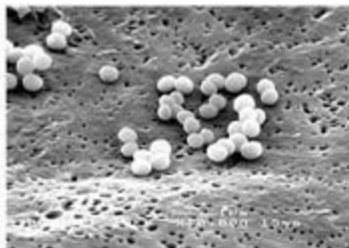


No physical changes seen on the bacterial cells after treatment with Gengigel®

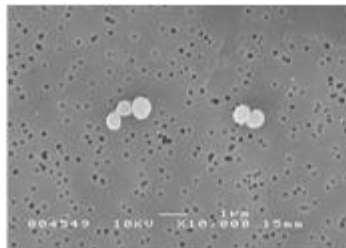


(Magnification: x10000, 10KV)
The bacterial cells shrank and formed irregular shapes after treatment with Chlorine Dioxide gel

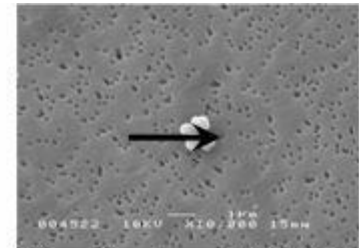
Figure 1. Scanning electron micrographs of *S. mitis* before and after treatment with Gengigel® and Chlorine Dioxide gel. *The arrows show the irregular shapes of bacteria after treatment with Chlorine Dioxide gel.



S. constellatus before treatment with Gengigel® and Chlorine Dioxide gel



No physical changes seen on the bacterial cells after treatment with Gengigel®



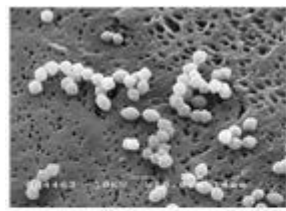
(Magnification: x10000, 10KV)
The bacterial cells shrank and formed irregular shapes after treatment with Chlorine Dioxide gel

Figure 2. Scanning electron micrographs of *S. constellatus* before and after treatment with Gengigel® and chlorine dioxide gel. *The arrow shows the bacterial shrinkage after treatment with chlorine dioxide gel.

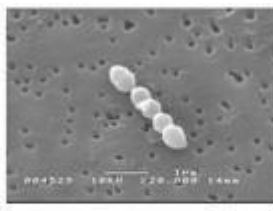
retain the smooth surface. *F. nucleatum* shrank, the cells surface wrinkled and the cell wall ruptured after treatment with ClO₂ gel (Figure 4).

DISCUSSION

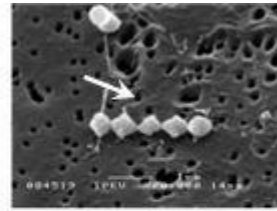
In this study, the antibacterial activities of Gengigel® and



E. corrodens before treatment with Gengigel[®] and Chlorine Dioxide gel



No physical changes seen on the bacterial cells after treatment with Gengigel[®]



(Magnification: x20000, 10KV)
The bacterial cell wall ruptured and the cell contents outburst after treatment with Chlorine Dioxide gel

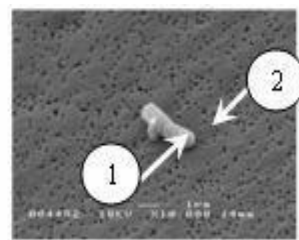
Figure 3. Scanning electron micrographs of *E. corrodens* before and after treatment with Gengigel[®] and Chlorine Dioxide gel. *The arrow shows the bacterial cell content outburst into the surroundings after treatment with chlorine Dioxide gel.



E. corrodens before treatment with Gengigel[®] and Chlorine Dioxide gel



No physical changes seen on the bacterial cells after treatment with Gengigel[®]



(Magnification: x20000, 10KV)
The bacterial cell wall ruptured and the cell contents outburst after treatment with Chlorine Dioxide gel

Figure 4. Scanning electron micrographs of *F. nucleatum* before and after treatment with Gengigel[®] and chlorine dioxide gel. * The first arrow shows the bacterial cell wall ruptured and the second arrow shows wrinkled surface of bacterial cells.

ClO₂ gel were evaluated on certain dental biofilm bacteria and also on pool samples of dental biofilm bacteria, both aerobically and anaerobically. Two methods were adopted to evaluate the antibacterial activities of these agents which were disk diffusion technique and MIC test. The disk diffusion technique was done to screen the antibacterial activities of the Gengigel[®] and ClO₂ gel, meanwhile MIC test was used as the main antibacterial test to evaluate the antibacterial activity. This test can determine the lowest concentration of antimicrobial agent needed to inhibit visible (99%) growth of bacteria after an overnight incubation (Brooks et al., 2004).

Initial screening with disk diffusion test showed positive results in which both Gengigel[®] and ClO₂ gel possessed antibacterial activities against all the microorganisms tested. In disk diffusion technique, clear zone of no bacterial growth formed surrounding the disk impregnated with agent on the MH agar surface. This clear zone was regarded as inhibition zone. The agent diffused into the MH medium, causing a zone of growth inhibition of the bacterial strain around the disk corresponding to the susceptibility of the bacteria strain to the agent.

Interpretative inhibition zone diameters have been established for susceptibility test results to permit classification of a bacteria as being susceptible, intermediate (decrease susceptibility) or less susceptible to an agent (Brooks et al., 2004).

The largest diameter of inhibition zone was produced by *S. mitis*, followed by *S. constellatus* and *S. aureus*. It showed that gram-positive bacteria were the most susceptible to Gengigel[®] and ClO₂ gel compared to Gram-negative bacteria and pool samples of dental biofilm. *F. nucleatum* proved to be the least susceptible bacteria by producing smallest diameter of inhibition zone compared to other experimental microorganisms tested. This particular bacteria is a gram-negative spindle form bacteria, which grows in an anaerobic environment and considered as the most pathogenic microorganism. Dental biofilm pool sample (anaerobic) also produced smaller inhibition zone compared to the other bacteria tested because it consists of various anaerobic bacteria species with different resistibility. *F. nucleatum* and dental biofilm pool sample (anaerobic) showed smaller diameter of inhibition zones due to their environmental conditions where both

microorganisms are anaerobic bacteria, which grows only in the presence of carbon dioxide and were slow grower (Willet et al., 1991).

MIC test was used to determine the antimicrobial activity of an agent against specific bacteria. Based on the results obtained from this study, the first microorganisms to be inhibited at the lowest ClO₂ gel concentration were *S. mitis*, *S. constellatus* and *S. aureus*. These three bacteria had lower MIC value (0.02% w/v) compared to the other bacteria tested, therefore confirmed that gram-positive cocci bacteria were the most susceptible bacteria towards ClO₂ gel. *E. coli*, *E. corrodens*, and *F. nucleatum* which belongs to gram-negative bacteria had higher MIC value (0.2% w/v) and less susceptible to ClO₂ gel.

Differences in the bacterial cell wall composition formed the basis for the explanation of why gram-positive bacteria were more susceptible to ClO₂ gel compared to gram-negative bacteria. The type of bacterial cell wall plays a crucial role. The cell envelope of gram -positive bacteria was relatively simple, consisting of two to three layers, cytoplasmic membrane, a thick peptidoglycan layer and some bacteria have an outer layer, either a capsule or S-layer. Antibacterial agents can easily penetrate through the peptidoglycan layer and destabilize the membrane permeability, thus interfering with the cell functions (Jack et al., 1995).

In contrast, gram-negative bacteria have a complex and multilayer structure. The cytoplasmic membrane, so called inner membrane, is surrounded by a thin layer of peptidoglycan which is anchored to a complex layer of lipopolysaccharide called outer phospholipidic membrane. The space between the inner and outer membrane is termed as periplasmic space (Brooks et al., 2004). This characteristic makes the cell impermeable to lipophilic solutes, while porins constitutes a selective barrier to the hydrophilic solutes. More over, the gram-negative bacteria have an active efflux mechanism that enables the bacteria to resist antimicrobial action (Stuart, 1992). Therefore, with these special features, the gram-negative bacteria were more resistant to ClO₂ gel.

The high resolving power of SEM has enabled the three dimensional images of the bacterial structure to be observed. The morphological structures of *S. mitis*, *S. constellatus*, *E. corrodens* and *F. nucleatum* were observed under SEM before and after treatment with Gengigel[®] and ClO₂ gel. This study did not detect any physical changes occurring in shapes and structures of the bacteria after treatment with Gengigel[®]. Therefore, it explains the results of MIC test done earlier in which Gengigel[®] showed no antimicrobial effects. The concentration of this commercially available Gengigel[®] is only 0.2% w/w, unable to inhibit visible growth of dental biofilm bacteria *in vitro*. Thus, we can say that the concentration of Gengigel[®] recommended by the manufacturer cannot give rise to any antibacterial effects hence shows no effect if it is being applied on dental biofilm, corresponds

to its main action as anti-inflammatory agent, not an antimicrobial agent (Xu et al., 2004).

Conversely, the scanning electron micrograph of bacteria after treatment with ClO₂ gel showed visible changes in the shapes and structures of the bacteria. ClO₂ gel caused shrinkage of most of the bacteria and form wrinkled surfaces. In some bacteria such as *F. nucleatum*, there was ruptured in cell wall, which then makes the cell content to outburst. The exact mechanism of action on how ClO₂ gel inactivates bacterial cells remains unclear. There were two possible explanations on how ClO₂ gel has inactivated microorganisms including bacteria and viruses. The first mechanism of action was the interaction of ClO₂ gel with specific biomolecules, and the second mechanism of action was the effect of ClO₂ gel on the microorganisms' physiological functions (EPA, 2002).

Generally, the antibacterial effect of ClO₂ gel was far greater than Gengigel[®] towards certain dental biofilm, bacteria and pool sample of dental biofilm, in accordance with all the antibacterial tests done and assessment of bacterial morphology under SEM. These findings further support the results reported by Yates et al. (1997) which showed that ClO₂ based mouthwash had equivalent plaque inhibitory action as Chlorhexidine. It is suggested that the exact mechanism of action on how ClO₂ inactivates bacterial cells to be investigated in future studies. Further investigation and experimentation of ClO₂ as the main constituent to form a gel preparation for use in clinical practice as an adjuvant to surgical and non-surgical periodontal therapy in chronic periodontitis patients is strongly recommended.

Conclusion

One of the more significant findings to emerge from this study is that ClO₂ gel has stronger antibacterial action against dental biofilm, bacteria compared to Gengigel[®]. For that reason, ClO₂ gel can be proposed as a good alternative ingredient for development of professional gel in order to control and inhibit various types of dental biofilm, microorganisms.

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