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Comparison of IPMP, Chlorine Dioxide and Chlorhexidine Gluconat Contained in Mouthwashes for Reducing Exopolysaccharide on Streptococcus Mutans Biofilms

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ABSTRACT

Background: Streptococcus mutans is the main bacteria forming biofilms associated with dental carious lesions. S. Mutans utilizes dietary carbohydrates to rapidly synthesize exopolysaccharides (EPS) using glucosyltransferase and fructosyltransferase enzyme. Purpose: This research aimed to assess the effects of three active ingredients of mouthwashes on EPS formed on S.mutans biofilms and to determine which ingredient is the most effective in reducing EPS. Method: This research is an experimental laboratory study with post test only control group design using EPS formed on Streptococcus mutans biofilms. EPS then was fluorescently labelled using alexa fluor 647 dextran conjugate stain and analyzed using Confocal Laser Scanning Microscopy and Fluoview ver 1.7a Software. Results: There is a significant difference between those three active ingredients of mouthwashes in reducing EPS and IPMP significantly eliminates EPS. Conclusion. IPMP has the most potential in eleminating EPS formed on Streptococcus mutans biofilms compared to other active ingredients.

INTRODUCTION

Dental caries is a disease commonly found throughout the world, both in children and adults. Although dental caries is not a threatening disease, it is still considered as the most common problem found by health workers (Forssten *et al*, 2010). Dental caries or cavities, can be caused by bacterial activity in dental plaque. There are three stages in the formation of dental plaque. The first is the absorption of proteins in the saliva by enamel even as soon as the teeth are cleaned. The tooth surface is covered by a complex combination of salivary proteins (glycoprotein, acidic proline-rich protein, and mucin), debris from bacteria, as well as sialic acid. The second stage is the occurrence of bacterial interactions with the pellicle formed through cell to surface interaction. Oral streptococcus is the first colony that attaches to the cleansed surfaces of the teeth, and forms biofilms within 4-8 hours. Biofilms are a collection of strongly attached microorganisms that produce extracellular polymeric matrices and are enveloped by carbohydrates (Samaranayake 2012). Microorganisms then will stay alive more by forming colonies and attaching to the surface of solids than hovering in liquid or plantonis. In the third stage, other bacteria, such as lactobacilli, attach to the first colony through cell to cell interaction (Marsh 2004, Decker *et al*, 2014)

Furthermore, various carbohydrates can be exploited by bacteria found in dental plaque, especially S. mutans, to be converted into a cariogenic energy source. Sucrose is a disaccharide that is not only fast to be fermented into acidic products, but also one of the carbohydrates that can be converted by bacteria into exopolysaccharide (EPS), one of the extracellular polymeric matrix composition (Forssten *et al* 2010, Samaranayake 2012, Decker *et al* 2014). EPS can affect dental caries development in several stages. First, polysaccharides provide food reserves. Second, EPS improves attachment. Third, EPS serves as a diffusion barrier while maintaining acid on tooth surfaces. Fourth, EPS increases plaque thickness while extending acid retention time (Samaranayake 2012).

Biofilm control is usually performed using mechanical cleansing and additional anti-microbial agents to prevent plaque mediated-disease, such as dental caries (Malic *et al* 2013). Mouthwash, for instance, is recommended as an antimicrobs, topical anti-inflammatory, or anti-caries. The anti-caries effects of mouthwash actually depends on several factors such as psychochemical properties inhibiting demineralization and accelerating remineralization as well as anti-bacterial agents inhibiting both the metabolic activity of Streptococcus mutans and the development of cariogenic bacteria in biofilms with low pH (Antonio *et al* 2010). The main ingredients of new mouthwashes are sodium chlorite also known as stabilized chlorine dioxide. Besides sodium chlorite, there is also IPMP (3-methyl-4-isopropylphenol / P-Thymol / 0-cymen-5-0l). IPMP is an active ingredient mouthwash known to have extensive antibacterial power. In addition, moutwashes still widely used until now contain chlorhexidine, known as "gold standard" for oral antiseptics.

MATERIALS AND METHOD

Rejuvenation of Bacteria

Streptococcus mutans bacteria were cultured on Brain Heart Infusion Broth (BHIB) media and incubated for 2 hours. Then 0.3 ml of the bacterial culture was taken with micropipet, and planted on Tryptone Yeast Cystein (TYC) medium. The bacteria were flattened by means of a spreader, and then inserted in the exicator or anerobic jar for 2 x 24 hours. Lastly, the bacterial colonies formed were taken and then planted on a medium containing Trypticase Soy Broth (TSB) and glucose to grow their biofilms.

EPS Biofilm Test Procedure

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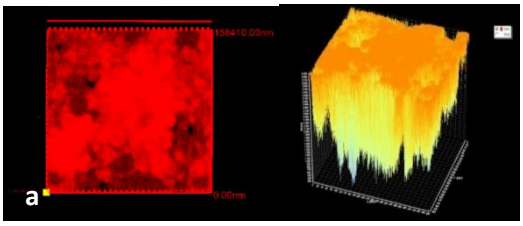
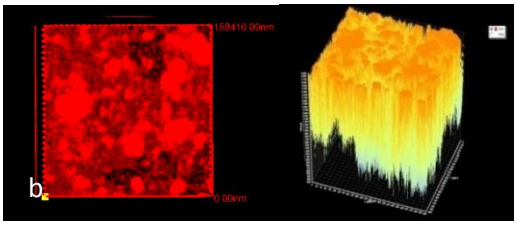
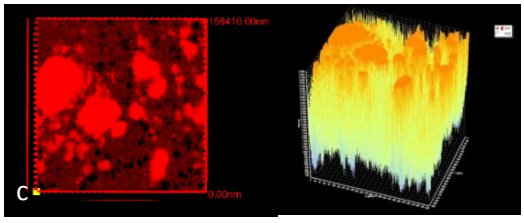
The S. mutans cultures that had been incubated for 1 x 24 hours on TSB medium were taken as much as 150 µl, and then inserted in microtiter petriplates (24 wells). 30 µl of Alexa Fluor 647 Dextran Conjugate Stain was added, they were incubated aerobically at 33° C for 24 hours. When biofilms were formed, 100 µl of a mouthwash ingredient (IPMP, CD, or CG) was added based on the type of the treatment and then shaken using IKA Microtiter Plate Shaker for 60 seconds at 500 rpm. They were cleaned using micropipette and fixated using PBS. The structure of the S. mutans biofilms was examined using on Confocal Laser Scanning Microscopy with a magnification of 80 x and a wavelength of 633 nm. Five horizontal pieces (XYZ orientation: horizontal pieces at 512 x 512 pixels) were taken from the biofilm on each petri plate. And the last, the images were analyzed using Fluoview ver 1.7a Images Analysis Software.

RESULTS

EPS formed on Streptococcus mutans biofilms in the control group, the group treated with mouthwashes containing IPMP, the group treated with mouthwashes containing CD, and the group treated with mouthwashes containing CG was measured using CLSM with alexa fluorine 647 dextran conjugate. Based on the results of the EPS measurement, the mean and standard deviation of EPS in the control group was 3258.965 + 209.361, 1225.256 + 449.768 in the group treated with mouthwashes containing IPMP, 3116.405 + 536.830 in the group treated with mouthwashes containing CD, and 2752.704 + 430.231 in the group treated with mouthwashes containing CG.







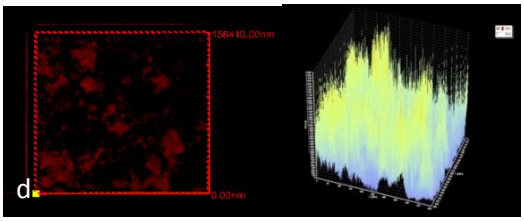


Fig.1: (a). The control group, (b).The group treated with mouthwashes containing CG (c). The group treated with mouthwashes containing CD. (d).The group treated with mouthwashes containing IPMP

Table 1. Comparison of the reseach groups

	C	CG	CD	IPMP
C		.053	.875	.000 *
CG			.239	.000 *
CD				.000 *
IPMP				

Note : * = significant

Based on results of the post-hoc test, it is known that mouthwashes containing IPMP significantly reduced EPS formed on Streptococcus mutans biofilms compared with other treatment groups using mouthwashes containing stabilized chlorine dioxide and chlorhexidine gluconate as well as the control group ($p < 0.05$). Mouthwashes containing chlorhexidine gluconate also decreased EPS, but the difference with the control group is not significant with the group treated with mouthwashes containing Stabilized chlorine dioxide ($p > 0.05$). Additionally, mouthwashes containing Stabilized chlorine dioxide also has no significant difference in reducing EPS compared with the control group ($p > 0.05$).

DISCUSSION

Biofilms are known as a collection of microorganisms that adhere strongly to the surface and form extracellular matrices, such as extracellular polysaccharides (EPS), proteins, and nucleic acids (Klein *et al* 2015). The formation of extracellular polysaccharide (EPS) matrix is actually related to the asidogenic properties of Streptococcus mutans in the presence of sucrose, glucose and fructose. EPS is also known as a long polymer chain with high molecular period.

The formation of dental plaque by Streptococcus mutans bacteria, moreover, is also influenced by glucosyltransferase (Gtf) produced by S. Mutans, then combined with glucan binding protein (GBPs). Gtf, responsible for forming glucan from sucrose, has an important role for the virulence of dental plaque (Krzyściak *et al* 2014). Glucan formed then provides binding site to form bacterial colonization and accumulation on the apatite surface as well as to bind each other through the interaction of membrane associated glucan-binding proteins and surface-glucans. The increased number of EPS, caused by the Gtf response to pH and the availability of carbohydrates, can enhance the virulence of biofilms. EPS may also affect the development of dental caries in several stages. First, Polysaccharides provide food reserve. Second, EPS improves attachment. Third, A water-insoluble EPS serves as a diffusion barrier while maintaining acid near the tooth surface. Lastly, EPS increases plaque thickness while extending acid retention time.

Moreover, biofilm controls to prevent plaque-mediated disease, such as dental caries, can be performed by mechanical cleaning as well as by the use of anti-microbial agents. For instance, mouthwash is recommended as an anti-microbial agent that can prevent dental caries. The anti-caries effects of mouthwash actually depends on several things, such as psychochemical properties inhibiting

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demineralization and accelerating remineralization as well as anti-bacterial agents inhibiting both the metabolic activity of Streptococcus mutans and the development of cariogenic bacteria in biofilms with low pH (Krzyściak 2014).

Therefore, this research used a method from Sun *et al.* (2014) to analyze the potential of three mouthwash products in Indonesia in reducing EPS formed on Streptococcus mutans biofilms. The results of this research were obtained by reading EPS, which was colored with alexa fluor 647 dextran conjugate, using Olympus Fluoview FV1000 CLSM (Confocal Laser Scanning Microscope). However, this research only focused on the active ingredient of the mouthwash products since the additives contained only have effects of prolonging the active period of the mouthwash products with different activities, not on bacteria (Ratcliff 2011).

The Tukey HSD test also revealed that there was a less significant difference in EPS formed on Streptococcus mutans biofilm between the group treated with mouthwashes containing chlorhexidine gluconate and the control group. This may be caused by the working mechanism of the mouthwashes containing chlorhexidine gluconate by binding with mucin in the saliva, thus reducing pellicle formation and inhibiting bacterial attachment on to the surface of apatite (Kalesinskas 2014). Besides, chlorhexidine also binds to bacteria, either plantonis or ones embedded in biofilms, leading to cell membrane rupture then causing leakage, in which the content of the bacterial cells is secreted or the bacterial cells dead (Oluremi 2011). As a result, it can be said that chlorhexidine works by blocking the formation of Streptococcus mutans microcolonies and damaging the membrane of the bacterial cells, thus blocking the bacteria to form EPS in the presence of glucose or sucrose. Similarly, Fabbri *et al* (2016) also argues that the biological activity of chlorhexidine has more dominant bactericidal effect than in degrading glucan or inhibiting EPS formation. Moreover, the results of this research also showed that there was not significat difference in decreasing EPS formed on Streptococcus mutans biofilms between the group treated with mouthwashes containing stabilized chlorine dioxide and other treatment groups. This may be due to the ability of stabilized chlorine dioxide to block the transport of nutrients through the cell membrane of bacteria, thereby suppressing the formation of exopolysaccharide by bacteria (Yadav 2015). Similarly, a research conducted by Sipros *et al* in 2008 stated that chlorhexidine is more significant in inhibiting plaque growth when compared to stabilized chlorine dioxide (Paraskevas 2008).

CONCLUSION

There is a potential difference between IPMP, Stabilized Chlorine dioxide, and chlorhexidine gluconate contained in reducing EPS formed on Streptococcus mutans biofilms. Additionally, it is also known that mouthwashes containing IPMP have the greatest potential to decrease the number of EPS formed on Streptococcus mutans biofilms when compared with other ingredients of mouthwashes, such as stabilized chlorine dioxide and chlorhexidine gluconate.

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