Original Article

Inhibition of Streptococcus Sanguis and Streptococcus Mutans in Gingivitis as a Result of The Mechanism of HNP1-3 and hBD-2

Hans Lesmana[1] , Harun Achmad[2] , Rini Sitanaya[3*] , Surya Irayani[4] , Ardian Priyambodo[5] , Ira Liasari[6], Ellis Mirawati[7]

[1,3,4,5,6,7]Dental Nurse Department Polytechnic of Health, Makassar, Indonesia

[2]Pedodontic Department, Hasanuddin University, Indonesia

Email: rinisitanayadrg96@gmail.com

Abstract

Gingivitis is a form of abnormality in periodontal tissue which can be found in the general society. The patient does not realize the clinical difference in gingiva caused by lack of knowledge about dental health and also there has not been a single case of mortality because of gingivitis Sickness of periodontal tissue is experienced by almost all people across the globe and it reaches 50% from total adult population. Parasympathetic stimulus when mastication is crucial to increase the velocity of saliva's flow, which will stimulate the polypeptide to show. In human, defensins polypeptide has two subfamilies, the Alpha defensins (HNP1-3) and Beta defensins (hBD-2) which interact with different targets, meanwhile hBD-2 specially interacts with lipopolysaccharide (LPS).

This research aim to explain the connection between mastication with increased content of hBD-2, HNP1-3and also the decrease amount of Streptococcus sanguis bacteria on gingivitis.

The method of this research is experimental with posttest design observation to the patient of gingivitis. The subject of this research are 42 man from 17-22 years of age, devided to 3 groups. Each group consists of 14 men (healthy group-mastication; group of gingivitis-mastication; group of gingivitis-no mastication). Mastication used standard gums (equal size, without sweetener and flavor) and it is done for a week. Every time subjects wake up in the morning and the mastication is done for one minute (32 times). The subject's saliva sample is contained in the Eppendorf Tube, which has been given Phenylmethylsulfonyl Fluoride (PMSF) 0,5 ml before.

The test result of Elisa content hBD-2 shows the striking difference of the three groups, HNP1-3 content only shows the difference in group 1:2 and group 1:3. Real Time Polymerase Chain Reaction (RT PCR) shows quantity of Streptococcus sanguis which showed the difference between group 1:3 and group 2:3

About mastication's role which caused the increase content of hBD-2 shows the meaningful relation in decreasing the quantity of bacteria is only to Streptococcus sanguis

Keywords: HNP 1-3, hBD-2, Streptococcus sanguis, Streptococcus mutans

Introduction

Gingivitis is inflammation of the gingiva, appears red, has normal contours, is swollen, bleeds with exudate and occurs after tooth eruption. Inflammation of the gingiva can occur in one or two teeth as well as in all teeth.

The gingiva bleeds easily with minor stimuli such as brushing your teeth, or without stimulation, and bleeding occurs all the time. Dental plaque bacteria in chronic gingivitis consist of 56% gram positive and 44% gram negative

Address for correspondence: Rini Sitanaya, Dental Nurse Department Polytechnic of Health, Makassar, Indonesia, Email: rinisitanayadrg96@gmail.com

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species, 59% species are facultative and 41% species are anaerobic. The dominant gram-positive species include Streptococcus Sanguis and Streptococcus Mutans. 1,2,3

In humans there are two subfamilies of defensins polypeptides, alpha defensins and beta defensins. Alpha defensin or Human Neutrophil Peptide is produced by polymorphonuclear leukocytes and consists of HNP1, HNP2, HNP3 and HNP4. Beta defensins or Human Beta Defensins are produced by epithelial cells, consisting of HBD-1, HBD-2 and HBD-3. Defensins is the first polypeptide antimicrobial discovered in humans. 4,5,6

Masticatory power causes the destruction of food, the magnitude of masticatory power in humans can be classified into four groups, namely: low with a strength of < 59 pounds, medium low with a strength of 59-100 pounds, medium high with a strength of 101 -144 pounds and high with a large strength greater than 144 pounds. The greater the mastication causes the secretion of more saliva. The research conducted showed that salivary secretion increased with high, medium high, medium low and low masticatory strength. The salivary secretion center that controls the amount of salivary secretion to be secreted is located in the autonomic nerves in the salivary glands. Stimulation of the parasympathetic nerves during mastication causes an increase in salivary secretion, and stimulates the release of polypeptides. 7,8,9

Animal studies have shown that the strength of mastication is determined by the type of food, recording can be done by electromyography. For example in experimental rats by feeding the type of grain or food that is hard or soft. Experimental rats given a harder diet, generally had thicker and stronger jaws than those given a soft diet, and the periodontal ligament was also thicker in the group of mice given a hard diet.8,10,11

Material And Method

This was an experimental study with observations on posttest design in patients with gingivitis. The population of this research was patients with gingivitis in the Dental Clinic of Dental Health of Polytechnic Makassar. The subjects research were 42 man of 17-22 years old, who not smoke, not takes medication of antibiotics at least for a month, not take any corticosteroid, don't have saliva abnormalities, not have blood abnormalities, and not have diabetes mellitus condition.

Obtain ethical clearance from the ethics committee of the Faculty of Medicine on the subject used as the research subject. Research explanations are given to all prospective research subjects, distribution of informed consent sheets to prospective subjects who will be subjects in the study, then filled out and signed as evidence of willingness to participate in the research.

There were 42 participants who were divided into 3 different groups which consisting 14 man. They were asked to fill out the question sheets and signed an informed consent. During the treatment there were six participants drop out, four participants drop out due to nausea after mastication in the morning. One person took antibiotics on the second day of the research period, and one person left without explanation.

The subjects were devided into 3 groups consisting 14 man. Group 1 is a group with healthy periodontium condition and will get a mastication treatment. Group 2 is a group with gingivitis and will get a mastication treatment. Group 3 is a group with gingivitis and will not get any mastication treatment. Mastication treatment used normal gums with equal size, without sweetener and flavor. The treatment was given for a week and must be conducted for a minute (32 times) in the morning right after the subjects wake up. The subject's saliva sample was collected after a week of treatment. After one week of treatment, all saliva samples was stored in an Eppendorf Tube that was given Phenylmethylsulfonyl Fluoride (PMSF) as a preservative of saliva. Saliva was examined for Alpha defensine (HNP1-3) and Beta defensine (hBD-2)levels, the number of Streptococcus mutans and Streptococcus sanguinis.

The results were statistically analyzed with SPSS application, using normality analysis, comparison test of Mann Whitney, and correlation test with Spearman

Protein Isolation Procedure

Protein isolated from the phenol-ethanol supernatant layer remaining after the DNA precipitation step. Isolating protein using either Protein precipitation or Protein dialysis: Protein precipitation, Protein washing, Protein resuspension, protein dialysis, Measure protein concentration by Bradford assay (SDS concentration should be <0, 1%).

Counting, levels of HβD-2, HNP1-3

The saliva samples obtained were added with detergent Without P-40 so that the concentration became 0.1% and then frozen. The samples obtained were then centrifuged twice at 15,000 rpm for 10 minutes. The concentration was calculated after the bicinchoninic acid assay was carried out in which an aliquot of 200 l of the supernatant was extracted with the addition of 1 M HCl/1% trifluoroacetic acid which was mixed in cold for 24 hours. After that the supernatant was concentrated by evaporation in a vacuum and then suspended in distilled water, then an ELISA test was carried out to determine the levels of HβD-2, HNP 1-3

Real Time Polymerase Chain Reaction Test (RT-PCR)

DNA extraction method using celite and guanidium thyocyanate procedures. The diatom suspension was prepared by adding 50 ml of water of 32% (w/v) Hcl or 445µ of 36% HCl into 10 grams of high purity analytical grade Celite. The diatom suspension was divided into several sterile tubes with a capacity of 2 ml, each containing 0.5 ml. The aliquots tube

was tightly closed and stored in a box in a sterile room, 20µl of this suspension would combine with 10µg of bacterial DNA. Increasing the volume of diatoms will increase the yield of DNA extraction. Diatoms do not need to be sterilized because the HCl contained in a diatom solution can damage the DNA of bacterial cells.

At the end of the procedure, a small amount of diatoms (about 1µl of diatom suspension in 100 ml of elution buffer TE) is obtained. The extraction results can be stored at -20C or -80°C (Powledge, 2005; Takara, 2005). The process of amplification of the PCR machine in a volume of 25µl with 2mM MgCl2; 0.2mM dNTPs; 0.1 g DNA extraction. Amplification was carried out by denaturing for 3 minutes at a temperature of 94°C, for 15 seconds.

Results

The effect of mastication on increasing levels of hBD-2, levels of HNP 1-3, decreasing the number of S.mutans and the number of Streptococcus sanguis.

Table 1: Research Data Descriptions

Variable	*Group 1*	*Mean*
hBD-2	Healthy Mastication	1089.08±320.80867
	Masticatory Gingivitis	640.357±410.34116
	Non Masticatory Gingivitis	318.618±228.19512

The mean of the HBD-2 group showed the highest consecutive values of the healthy, masticated group; masticatory gingivitis group; and non masticatory gingivitis group

Table 2: Description of research data

Variable	*Groups*	*Rank*
HNP1-3	Healthy Mastication	33.36±4.105783
	Masticatory gingivitis	18.18±4.105783
	No Masticatory gingivitis	12.96±4.105783
S sanguis	Healthy Mastication	14.75±73913.3063
	Masticatory gingivitis	17.82±73913.3063
	No Masticatory	31.93±73913.3063
S mutans	Healthy Mastication	22.86±28.028706
	Masticatory gingivitis	19.5±28.028706
	No Masticatory gingivitis	22.14±28.028706

The mean of the HNP1-3 group showed the highest consecutive scores of the healthy, masticated group; masticatory gingivitis group; and no masticatory gingivitis group. The mean of the S sanguis group showed the lowest consecutive values of the healthy group, mastication; masticatory gingivitis group; and no masticatory gingivitis group. The mean of the S.mutans group showed the lowest value of the gingivitis, masticatory group; In the healthy group, mastication was almost the same as the non-masticating gingivitis group.

Normality test on human Beta Defensin 2 (hBD-2), Human Neutrophil 1-3 (HNP 1-3), Streptococcus sanguis and Streptococcus mutant variables with the One Sample Kolmogorov-Smirnov test (sample less than 50) and the results are: all not normally distributed, except for hBD-2. To analyze the differences in hBD-2 of the 3 groups, the ANOVA test was carried out, then continued with post-ANOVA using LSD (if the assumption was fulfilled that the variance was balanced, equal) and using the Tamhene test if the assumption of the same variance was not met. The results of the ANOVA test showed that the variance of the 2 variables (using Laventes stat) had the same variance, $p > 0.05$ so that the ANOVA test could be continued. And the results of the Anova test can be concluded that there is a significant difference between the 3 groups in hBD-2 . levels

Table 3. The results of post-Anova test differences in hBD-2 levels

Healthy Mastication Group	Masticatory Gingivitis	0,001
Healthy Mastication	No Masticatory Gingivitis	0,000
Masticatory Gingivitis	No Masticatory Gingivitis	0,013

Meaningful if p<0,05

Based on table 3, the levels of hBD-2 which were analyzed by ELISA method in the three groups compared all showed a significant difference (p≤ 0.05), the masticatory healthy group compared to masticatory gingivitis (p = 0.001), the masticated healthy group compared to no-masticatory gingivitis (p = 0.001). p = 0.000), and masticatory gingivitis compared to no-masticatory gingivitis (p = 0.013)

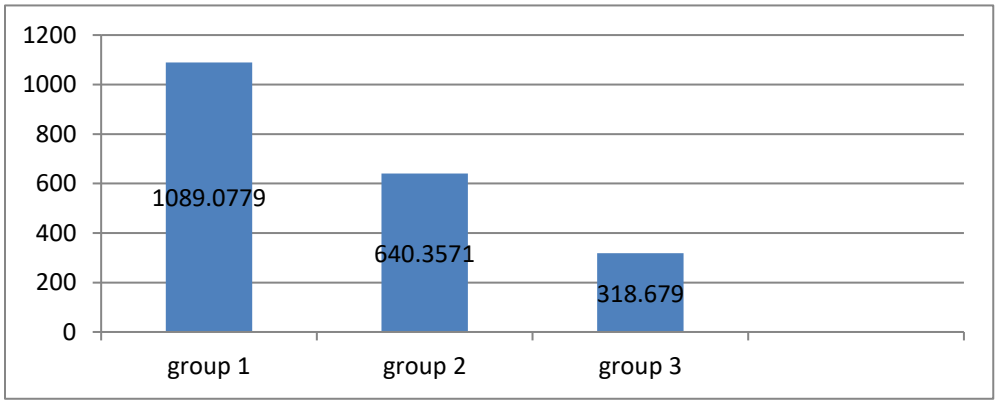

Figure 1. Levels of hBD-2 (ug/ml) in the healthy group of masticatory, masticatory gingivitis and no masticatory gingivitis

To analyze the differences in HNP1-3, S sanguis and S mutants from the 3 groups, the Mann-Whitney Test was carried out.

Table 4. Difference test with Mann Whitney on HNP1-3

Healthy Mastication Group	Masticatory Gingivitis	0,000
Healthy Mastication	Non Masticatory Gingivitis	0,000
Masticatory Gingivitis	Non Masticatory Gingivitis	0,246

Information : meaningful if p<0.05

Based on table 4, HNP1-3 levels analyzed by ELISA showed that the masticated healthy group compared to the masticatory gingivitis group showed a significant difference (p = 0.000) and the masticated healthy group compared to the no masticatory gingivitis group was also significant (p = 0.000). Only comparison of masticatory gingivitis group with non masticatory gingivitis showed no significant difference (p = 0.246).

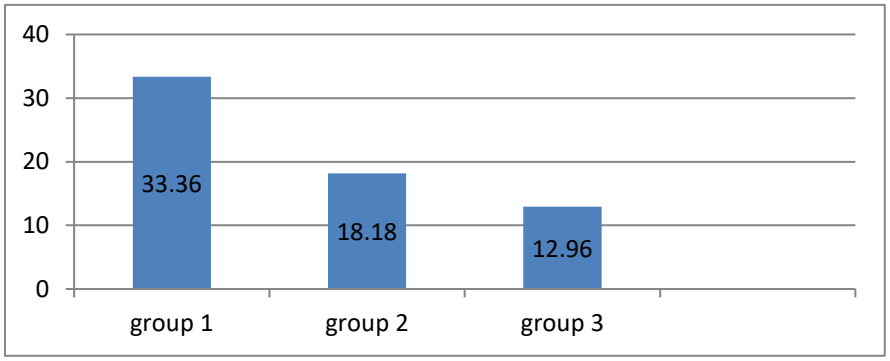

Figure 2 Levels of HNP1-3 (ug/ml) in the healthy group of masticatory, masticatory gingivitis and non-masticating gingivitis

Table 5. Test differences with Mann Whitney on S sanguis

Healthy Mastication Group	Masticatory Gingivitis	0,603
Healthy Mastication	Non Masticatory Gingivitis	0,000
Masticatory Gingivitis	Non Masticatory Gingivitis	0,003

Information : p <,0,05

Based on table 5, the number of Streptococcus sanguis bacteria compared, healthy masticatory compared to masticatory gingivitis did not show a significant difference (p = 0.603), while healthy masticatory compared to non-masticating gingivitis (p = 0.000) and masticatory gingivitis compared to non-masticating gingivitis (p = 0.003) show a significant difference.

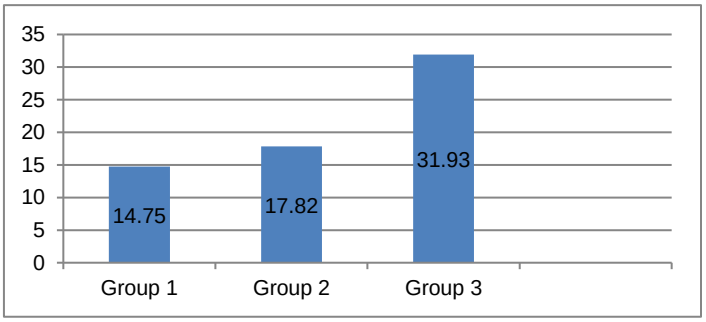

Figure 3. The number of Streptococcus sanguis (CFU/ml) in the healthy group of masticatory, masticatory gingivitis and no masticatory gingivitis

Table 6 Difference test with Mann Whitney on S mutant

Healthy Mastication Group	Masticatory Gingivitis	0,427
Healthy Mastication	Non Masticatory Gingivitis	0,946
Masticatory Gingivitis	Non Masticatory Gingivitis	0,635

Information : meaningful if p<0,05

Based on table 6, the number of mutant S bacteria in the three groups which were compared all showed no significant difference, masticatory health compared to masticatory gingivitis (p = 0.427), healthy masticatory compared to non-masticating gingivitis (0.946) and masticatory gingivitis compared to non-masticating gingivitis (0.635).).

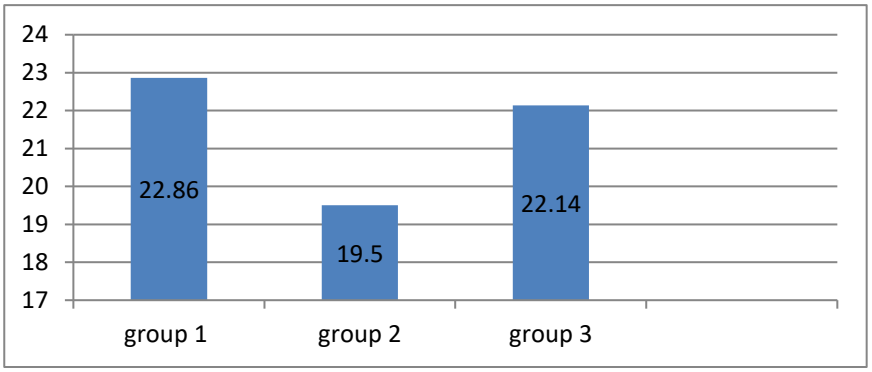

Figure 4. The number of mutant Streptococcus bacteria (CFU/ml) in the healthy group of masticatory, masticatory gingivitis and non-masticizing gingivitis

Table 7 Spearman Correlation Test for levels of hBD-2, HNP1-3 on the number of Streptococcus sanguis and Streptococcus mutans

variabel	*Streptococcus sanguis*	*Streptococcus mutan*
hBD-2	p= 0,014	p= 0,561
HNP1-3	p= 0,114	p= 0,546

* p < 0,05

Based on table 7, it is stated that increasing levels of hBD-2 (p=0.014) correlated with a decrease in the number of S sanguis bacteria, and HNP 1-3 (0.114) did not correlate with a decrease in the number of S sanguis bacteria. Increased levels of hBD-2 (0.561), levels of HNP1-3 (0.546) did not correlate with a decrease in the number of mutant S bacteria.

Discussion

Mastication not only has an impact on oral health, but also on systemic health. Mastication can increase heart rate and blood pressure, cortisol, and cerebral vascular flow. Mastication with unsweetened gum increases brain glucose which is associated with increased metabolic activity. Research using humans as samples provides strong evidence that samples with masticated unsweetened chewing gum have a higher increase in cognitive function than samples that do not masticate. The masticatory effect of chewing gum containing xylitol has an impact on plaque reduction. Xylitol weighing 3 grams did not have a significant effect on plaque reduction, while xylitol weighing 6 grams and 9 grams had a significant effect on plaque reduction.9,10

Mastication increases salivary secretion through a stimulated salivary reflex that occurs when chemoreceptors or pressure receptors are in the oral cavity. These receptors initiate impulses in afferent nerve fibers that carry information to the salivary center in the medulla of the brainstem. The salivary center sends impulses to the extrinsic autonomic nerves to the salivary glands to increase salivary secretion. Masticatory movements stimulate the secretion of saliva even though there is no food due to manipulation of pressure receptors in the mouth.7,8

Correlation of Human Beta Defensins-2 (hBD-2) with Streptococcus Mutans and Streptococcus Sanguis

There was a significant difference in the hBD-2 levels in the comparison of all groups. Human Beta Defensin two (hBD-2) is produced in the sublingual gland, which produces 70% of the total saliva per day so that mastication greatly increases salivary secretion including the levels of hBD-2 present in it. Another study on animals showed that white rats that were given a salivary secretion stimulant gave a significant difference with white rats that were not given a salivary excitatory substance in the amount of polypeptide secretion in saliva. It has broad spectrum anti-bacterial properties, so it is induced by the presence of oral bacteria, both in bacteria. mutant Streptococcus and Streptococcus sanguis bacteria. Another study on hBD-2 that the induction of hBD-2 occurs by the release of chemokines by human oral epithelial cells (HOEC) inhibits the bacterium Fusobacterium nucleatunm which is an anaerobic bacterium in periodontitis Human Beta Defensin two can also inhibit the bacteria Helicobacter pylori, Haemophilus Parainfluenzae, Candida Albicans, hBD-2 bacteria gram positive and gram negative has the ability to create pores on the target bacterial cell membrane. 6,12

The ability of hBD-2 to induce CCL20 release was increased in the first 24 hours of plaque formation. Plaque formation in

the first 24 hours is dominated by gram-positive bacteria by Streptococcus Sanguis, other studies have shown that hBD-2 increases significantly in 20-24 hours. Expression at low levels of hBD-2 can inhibit gram-negative bacteria, the target of bacteria is a lipopolysaccharide (LPS). The target for gram-positive bacteria is teichoic acid, the common target for gram-positive and gram-negative bacteria is the bacterial membrane that contains a lot of phospholipids (phosphaticyl glycerol) . This study is in line with research which states that the expression of hBD-2 strengthens the antimicrobial activity of defensins to inhibit pathogenic bacteria, both in healthy and inflammatory subjects.5,13,14

Correlation of Human Neutrophil Peptide (HNP 1-3) with Streptococcus Mutans and Streptococcus Sanguis

Neutrophils have a very large number in normal conditions that reach 60-70%, but in an inflamed state it will increase rapidly to 90% and under the influence of several humoral factors and related cellular signals, neutrophils migrate to the site of infection which inhibits pathogens and damaged cells. Neutrophils in saliva are the most prominent line of defense of immune cells for defense against microbial pathogens.14 Neutrophils make an important contribution in the activation and recruitment of macrophages at sites of infection or acute inflammation. Neutrophil activation during infection increases the synthesis and release of various chemokines, such as macrophage inflammatory protein-1α (MIP-1α) which acts strongly against macrophages. Activated macrophages prolong the life span of neutrophils at sites of inflammation or infection. 15,16

Neutrophil recruitment requires adhesion and transmigration through the vessel wall at sites where the vascular endothelium is activated by proinflammatory mediators. Transendothelial migration is a directed movement of blood to inflamed tissues that is the result of selective interactions between neutrophils and endothelium that causes neutrophils to push the path between endothelial cells to leave the blood and enter the tissue. Abnormalities in transendothelial migration are associated with aggressive periodontitis. Neutrophils will be stimulated to exit through the gingival microvascular and then enter the periodontal tissue and then migrate towards the endogenous epithelium and serum-derived. 15,17

Human Neutrophil Peptide (HNP1-3) in saliva is the result of induction by various inflammatory and microbial stimuli. The mechanism of microbial activity of defensins is based on its cationic and amphipathic molecules. The constituent antimicrobial peptides bind to the negatively charged bacterial cell surface and form pores in the lipid bilayer thereby helping the cytoplasmic membrane and its permeability leading to bacterial inactivation.6,18

The content of HNP 1-3 produced in the sublingual gland (5%) from 500-1000 ml of the daily volume of saliva produced per day causes mastication to not produce a lot of HNP1-3 secretion. Based on the results of this study, it can be seen that in healthy conditions, mastication gave significant differences compared to the gingivitis, masticatory group and the gingivitis, non-masticating group. The masticatory effect caused a significant increase in HNP1-3 levels, but not significant in gingivitis. HNP 1-3 levels also did not inhibit the bacteria Streptococcus sanguis and Streptococcus mutans. 17,18

This study is in line with the findings in white rats that were given a drug to stimulate salivation, showing a significant release of antimicrobial peptides compared to mice that were not given a salivary-stimulating drug. HNP1-3 levels also did not have an impact on reducing bacteria because the subjects examined were in good health and not in acute inflammation, this is in line with research which states that HNP1-3 levels increase in active caries.4,5,19

Streptococcus mutans and Streptococcus Sanguis

The low number of Streptococcus Sanguis bacteria compared to Streptococcus Mutans bacteria in this study is in line with epidemiological research which shows that the presence of Streptococcus Sanguis bacteria that produces H2O2, in the early stages of colonization and in large numbers is significantly associated with low levels of Streptococcus mutans bacteria , whereas the number of S mutants that produce mutacins causes a decrease in the number of S sanguis bacteria.14,19

Biofilm is a very complex structure and is an ecosystem of oral bacterial colonies that compete cooperatively or competitively with one another. Biofilm communities are complex and dynamic structures that gather through colonization. several consecutive and regular oral bacteria. The most prominent feature of biofilms is that bacteria often express different phenotypes from planktonic bacteria. Many bacterial species in biofilms show greater tolerance to antibiotics and environmental factors, such as pH and oxygen.19,20

Biofilm is a polysaccharide matrix that covers a population of bacteria that adhere to each other and / or adhere to surfaces or between surfaces. The microbial stage in the formation of biofilms and the formation of the colonization environment is sometimes unclear, but initially film deposition occurs, this stage involves the absorption of inorganic molecules and organics on the solid surface and then leads to film formation. Film formation involves proteins and glycoproteins derived from saliva. Adhesion and colonization of planktonic microorganisms, at this stage the attachment is strengthened by polymer production and this stage starts from the cell surface. The organisms that pioneered the formation of biofilms were Streptococcus species followed by the next layer consisting of gram-positive and gram-negative bacteria. Bacterial growth and expansion of biofilms, monolayers of early colonizing microorganisms attract secondary colonization to form microcolonies. The interaction of two types of microbes is seen at the cellular level during the

formation of co-adhesion and coaggregation of biofilms.21,22

The erupting tooth is immediately protected by a thin layer of glycoprotein called the acquired pellicle. Glycoprotein from saliva is immediately absorbed by hydroxyapatite and then adheres tightly to the tooth surface. Early in plaque formation, aerobic bacteria are the first to adhere and reach more than 50% are Streptococcus Sanguis bacteria and are then followed by other bacteria. Initial attachment of these bacteria to hydroxyapatite is very weak and is reversible, so these bacteria do not form colonies. Streptococcus Mutans bacteria then synthesize extracellular dextran, causing attachment and aggregation of bacteria followed by increased colonization, by dextran receptors on the surface of the bacterial cell wall.15,21

Streptococcus Sanguis is able to synthesize extracellular dextran from sucrose in the form of 1-6 chains and is soluble in water. Streptococcus Mutans synthesizes more water-insoluble dextran with chains 1-3 so that this bacterium is stronger in colonizing plaque formation than Streptococcus Sanguis, this is in line with research on mastication that increases levels of hBD-2, HNP1-3 which shows that only Streptococcus Sanguis bacteria that can be significantly reduced by increasing levels of hBD-2 is different from Streptococcus Mutans which does not show a significant decrease by increasing hBD-2, HNP1-3.14,21,23

Metabolism of extracellular sucrose by mutant Streptococcus, with its water-insoluble chain 1-3 dextran products, plays an important role in the mechanism of dental plaque formation and increased colonization in plaque.26,27 This increase in colonization occurs due to bacterial aggregation through heterotypic attachment between cells and heterotypic attachment between different cells. Dextran with chain bonds 1-3 also act as aggregation mediators between mutant Streptococcus and Streptococcus Sanguis. 4,21

Streptococcus Mutans in plaque metabolizes sucrose to acid in a faster time than other bacteria. Streptococcus mutant colonies are covered by glucans or dextran which can reduce the antibacterial activity of saliva against dental plaque. Oral bacteria can withstand oxygen, host immunity and antimicrobial agents through the formation of biofilms as a barrier unit.22,224 Oral bacteria contained in the biofilm are more resistant to antimicrobials, because the biofilm matrix is less permeable to antimicrobials. The defense of the bacterial biofilm is related to the presence of a protective barrier provided by the extracellular polymer matrix (EPM). The biofilm bacteria showed higher resistance to antimicrobials compared to the free planktonic form. The influencing factors include; First, the presence of an extracellular matrix physically limits the diffusion of antimicrobial agents. Second, slow growth in biofilms contributes to antimicrobial resistance due to lack of sensitivity to growth-dependent antimicrobials. 5,24,28

Ethics

The ethical approval number of this study was 193/KKEPK.FKG/XII/2014.

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