REVIEW OF APPROACHES TO CARIES TREATMENT

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Abstract

Dental caries is considered to be one of the most prevalent infectious disease in humans across all age groups because they affect 60-90% of the world's population. With the introduction of refined sugar into the modern diet and an improved understanding of the pathogenesis of dental caries, the need for novel approaches to caries prevention has become is a key undertaking for addressing the caries burden in the 21st century. While previous studies have shown successful elimination of bacteria in the oral cavity, only a few modalities such as antimicrobial peptides, vaccines and more recently, nanoparticles, have been developed to specifically target Streptococcus Mutans. Here, we discuss the significance of the biofilm and the essential role of Strep. mutans in the development of dental caries. We focus on current and prospective therapeutic approaches against dental caries, including chemical modalities such as fluoride and chlorhexidine, vaccines, antimicrobial peptides, replacement therapy, quorum sensing targets, nanoparticles and natural products. Further research is needed to develop a safe and efficacious approach against caries, or specifically Streptococcus Mutans, in humans.

I. Discussion of National and Worldwide Burden of Caries

Dental caries is one of the most common childhood diseases. It is estimated that over 90% of the world's population will experience dental caries at least once during their lifetime (Marchisio 2010). Dental caries is considered to be one of the most prevalent infectious disease in humans across all age groups because they affect 60-90% of the world's population (Petersen 2005).

Oral diseases and disorders affect health and well-being throughout life. According to the Oral Health in America: A Report of the Surgeon General in 2000, dental caries is the single most common chronic childhood disease - 5 times more common than asthma and 7 times more common than hay fever. Over 50% of children 5-9 years old have at least one cavity or filling and that proportion increases to 78% among 17 year olds and 84.7% for adults 18 or older. Additionally, 49.7% of people 75 years or older had root caries affecting at least one tooth (NCHS 1996, NHANES III). By age 17, more than 7.4% of US children have lost at least one permanent tooth because of caries; by age 50, Americans have lost an average of 12.1 teeth, including third molars. Dental caries is one of the primary causes of tooth loss among adults.

The social impact of oral diseases is substantial in children, amounting to more than 51 million school hours lost each year due to dental-related illness. Disparities in dental disease can be attributed to socioeconomic status. (Surgeon General's Report 2000). More than 1/3 (36.8%) of poor children aged 2-9 have one or more untreated decayed primary teeth, compared to 17.3% of non-poor children. Furthermore, the proportion of teeth affected by dental caries also varies by age and race/ethnicity. Poor

children from Mexican-American or non-Hispanic black backgrounds are at a higher risk group, with over 2/3 of this population having untreated decayed teeth. (NCHS 1996).

In the African American population, “baseline data for the Healthy People 2010 objectives established that for children aged 2 to 4 years, 24.0 percent of non-Hispanic blacks have experienced dental caries in their primary teeth, compared to 15.0 percent for their non-Hispanic white counterparts.” (Surgeon General’s Report 2000). In the Asian and Pacific Islander American (APIA) population, “there is variation in oral health status among subgroups of children. In a recent survey in Hawaii, the prevalence of early childhood caries among APIA children was 16 percent, ranging from a low of 8 percent among Japanese children to a high of 25 percent among Filipino children. The prevalence of untreated dental caries in 6- to 8-year-old APIA children was 39 percent, which ranged from a low of 16 percent among Japanese children to 40 percent among Native Hawaiians, 48 percent among Southeast Asians, and 62 percent among non-Native Hawaiian Pacific Islanders (Greer 1999). (Surgeon General’s Report) In the Hispanic group, “preliminary data from NHANES III indicate that young Mexican American children aged 2 to 4 are more likely to have experienced dental caries in their primary teeth, have on average more decayed and filled tooth surfaces, and have more untreated disease than either non-Hispanic white or non-Hispanic black children (Kaste et al. 1996). Mexican American children aged 2 to 5 years—especially those from lower-income households—were more likely than their African American and non-Hispanic white counterparts to have one or more decayed primary teeth (Vargas et al. 1998). A national survey found that employed Hispanic adults were twice as likely to have

untreated dental caries as non-Hispanic whites.(Watson and Brown 1995)" (Surgeon General's Report 2000)

Healthy People 2020 has focused on 2 leading evidence-based interventions to prevent tooth decay: 1) Community water fluoridation and 2) School-based dental sealant programs. Community water fluoridation is an effective, safe and ideal public health measure which benefits individuals of all ages and socioeconomic status. Studies show that it can reduce tooth decay by 18-40%. However, over one third of the US population (100 million) do not currently benefit from this measure. (Surgeon General's Report 2000). According to statistics from the Centers of Disease Control in 2013, over 70% of US residents who rely on community water systems receive fluoridated water. CDC recognizes community water fluoridation as 1 of the 10 great public health achievements of the 20th century.

School-based dental sealant programs usually target schools that serve children from low-income families. Dental sealants can prevent up to 60% of tooth decay in the treated teeth. (Ahovuo-Saloranta A et al 2013). Thirty six states reported dental sealant programs serving 258,000 children. This represents only about 8% of lower income children who could receive sealants. Currently used strategies for caries prevention such as oral health education, chemical and mechanical plaque control, use of fluorides, application of pit and fissure sealants are mostly broadly effective. (Surgeon General's Report 2000)

According to the World Health Organization's Report on "Oral health: action plan for promotion and integrated disease prevention" in May 2007, future action calls for

further support for building at the national and community level, and implementing comprehensive and integrated oral-health programs, particularly in low and middle income countries and for poor and disadvantaged groups. In developing countries, dental caries remains in epidemic proportions. An oral health survey conducted in China revealed that 3 out of 4 5-year old children had evidence of significant dental decay. (Wong et al 2001). More effective public health measures are necessary to address this worldwide problem.

II. Understanding the Complexity of Biofilms

While the pathogenesis of dental caries is complex, it often begins with cariogenic bacteria entering the dental biofilm at the early stages of life and subsequently emerging, under favorable environmental conditions to cause disease. (Smith 2002). In 1993, the term “Window of Infectivity” was coined by Caufield to describe the observation that children become permanently colonized with Streptococcus mutans between the middle of their second year and the end of the 3rd year of life under normal diet and environmental challenge. If infants fail to acquire Streptococcus mutans (MS) during this period they are unlikely to be colonized until around 6 years of age when the permanent molar teeth erupt. The higher the level of maternal strep mutans, the higher the percentage of children who become infected. (Kohler et al 1984). Though studies have shown that the primary source of infection is maternal, a few studies have also suggested that non-familial transfer can occur when the environmental conditions favor colonization (Mattos-Graner et al 2001). One of the most important virulence factors of cariogenic bacteria is their ability to adhere to oral surfaces and the colonization of subsequent bacteria to create biofilms.

Biofilms are complex microbial communities. A recent report using pyrosequencing analysis estimated the number of phylotypes in the oral cavity to be greater than 19,000 (Keijser et al 2008). This matrix consists mainly of hydrated polysaccharides, proteins, glycopeptides, extracellular DNA and lipids (Dong et al 2002). A biofilm is usually polymicrobial, consisting of multiple organism species such as fungi, bacteria and viruses that exist at a phase or density interface, and encased in

a self-secreted extracellular matrix. The extracellular matrix which surround the bacteria reduces penetration of antimicrobial agents into the deeper layers of the biofilm. Cohabitation of multiple organisms not only creates competition for space but also promotes cooperative interactions such as horizontal gene transfer, metabolic cooperation and other synergies. This enhances the "survivability of microorganisms and resistance to antimicrobial agents" (Yang et al 2011; Wolcott et al 2013).

Since the biofilm responds as a community rather than as an assemblage of individual cells, cell-to-cell coordination, communication and signaling are imperative. Quorum sensing, the secretion and detection of a diffusible molecule by the community members can lead microbial species to suddenly change their behavior. One example is production of virulence factors (Remis et al 2010). There are 3 physiological properties of the biofilm that makes it an effective barrier that is difficult to overcome with therapeutics. (Hoiby et al 2010, Mah et al 2012) Firstly, there is a delayed or reduced penetration of any antimicrobials due repulsion and/or sequestration by the exopolysaccharide matrix. Secondly, the presence of metabolically inactive "persister" cells that are able to survive antimicrobial attacks, preventing complete elimination of the colony. Thirdly, due to close proximity of other cells, there is an increased possibility of exchange of mobile genetic elements encoding resistance. When cells exist in a biofilm, they can become 10-1000 times more resistant to the effects of antimicrobial agents. (Mah et al 2012) This presents a challenge in treatment targeting cariogenic bacteria which reside in the oral biofilm.

The molecular pathogenesis of biofilm formation involves several phases. A saliva film containing albumin, glycoproteins, acidic proline-rich proteins, mucins, silica acids, and other compounds cover the tooth surface. This film called the acquired pellicle, provides receptors for the initial colonizers (Kreth et al 2009). Initial attachment to the tooth surface is achieved via interaction of bacterial proteins with lectins in the dental pellicle covering the tooth surface. (Smith et al 2002) Streptococcal adhesins, also known as antigen I/II or PAc in Strep mutans, bind to salivary components in experimental dental pellicles. The accumulation phase of acidogenic streptococci is then initiated by the activity of extracellular glucosyltransferases (GTF). Specifically GTF-B and GTF-C have been found to synthesize insoluble forms of glucan which is most closely associated with pathogenicity. In the presence of sucrose, GTFs synthesize several forms of high-molecular weight branched extracellular glucans. These glucans act as a scaffold for the aggregation of Strep mutans or other bacteria through the interaction with bacterial cell-associated glucan-binding domains of GTFs and Glucan Binding Proteins (GBPs). The succession of biofilm development involves congregation and coadherence of oral bacteria, and if undisturbed, develops into a stratified, complex biofilm. (Kreth et al 2009) The last phase of pathogenesis is the production of lactic acid, a bacterial metabolic end product, which results in the erosion of hydroxyapatite-like mineral in dental enamel. It is critical to understand the intricacies of the dental biofilm in order to understand possible targets for anti-caries intervention.

The initial colonization process is dominated by oral streptococci, which make up over 80% of the early biofilm constituents (Rosan and Lamont, 2000) Current

approaches to biofilm control include either a therapeutic focus, with elimination of mature biofilms or a prophyolactic focus, preventing biofilm development by killing planktonic cells, disrupting their adhesion to a surface or inhibiting growth of attached cells in the early biofilm stage (Jorge et al 2012). Strep mutans fuse with dental pellicles via 2 independent mechanisms: sucrose-dependent and sucrose independent. The sucrose dependent pathway involves the expression of antigen I/II (Mitchell 2003) and a virulence factor which binds the glycoprotein which coats teeth, salivary agglutinin. The sucrose independent pathway is more efficient and depends on glucans for attachment to the tooth surface. GTFs B, C and D produced by Strep. mutans convert sucrose to glucans.

III. Historical Perspective of Cariogenic Bacteria

W.D Miller first proposed the acid decalcification theory of dental caries in 1890. This theory involved 2 steps. Firstly, acid is produced from fermentable carbohydrate by bacteria in the mouth. Secondly, the acid dissolves tooth mineral resulting in a cavity. Miller considered that all bacteria in the mouth were potentially cariogenic, a concept now known as the “non-specific plaque hypothesis”.

In 1986, Loesche coined “specific/non-specific plaque hypothesis” for dental caries. The specific plaque hypothesis stated that Strep mutans is the primary cause of dental caries and treatment should be directed toward its elimination. The non-specific plaque hypothesis on the other hand stated that treatment of caries should be directed toward the correction or compensation of any oral microbiota imbalance. Bacteria are necessarily secondary and the promotion of oral bacteria that produce alkali is crucial. Additionally, the non-specific plaque hypothesis suggests that oral bacteria as a whole produce acid from fermentable carbohydrates. Therefore, the removal of as much plaque as possible is the appropriate treatment in dealing with caries.

While Clarke first isolated Strep mutans from carious lesions in 1924, it was a keystone study by Fitzgerald and Keyes in 1960, where direct evidence for involvement of specific microorganisms in dental caries was observed in albino hamsters. These hamsters only developed caries when caged together with other “caries-active hamsters”. This showed that specific microorganisms were involved in the induction of dental caries and that the disease was transmissible. Several studies led to the discovery of at least 8 serotypes of Strep mutans, which were grouped mainly into 4

species relevant to humans and small laboratory animals. Serotypes c, e and f remained Strep mutans. Serotype c Strep mutans strains are most frequently isolated from the human oral cavity (Loesche 1986) Serotype d, g and h became Strep sobrinus. Serotype a became Strep cricetus and serotype b became Strep rattus. Collectively, they are referred to as Mutans Streptococci (MS). Strep mutans and Strep sobrinus the are the most important in dental caries. In addition to Strep mutans, Lactobacilli have also been found to have an associative relationship with caries development. (Kleinberg 2002) Though not essential in the caries process, lactobacilli have been found in sites favoring retention of fermentable carbohydrates or sites with dental appliances such as orthodontic bands. These sites have an elevated count of Lactobacilli and more acidogenic dental plaque.

In 2002, Kleinberg proposed the “mixed-bacterial ecological approach” as another model for caries development. This approach suggests that the proportion of acid-base producing bacteria is key in dental caries. Manipulating the proportion of arginolytic and non-arginolytic bacteria in the oral cavity changes the level of caries activity in an individual. Two landmark studies by Stephan in 1940 and 1944 demonstrate that dental plaque produced rapid and substantial decreases in the pH in vivo. pH would substantially decrease following a sugar challenge and then slowly rise to baseline over an hour. The effects of saliva on the Stephan pH curve was studied. In the presence of saliva, there was a fall in pH to a minimum, then a subsequent slow rise toward baseline. In the absence of saliva, pH dropped significantly and then remained at a lower level for much of the experimental period. The rise part of the Stephan curve

was lost in the absence of saliva, possibly due to the absence of salivary bicarbonate (Wijeyeweera et al 1989). Through fractionation experiments, Kleinberg identified the pH-rise factor in saliva as arginine. Arginolytic bacteria produce base from arginine. Major acidogenic bacteria are non-arginolytic; they produce a pH fall but little or no pH rise. Other acidogenic bacteria also produced a pH fall but one that was less, because these bacteria produce base from arginine (arginolytics). Bacteria in plaque that are able to raise the pH by degrading arginine and producing base would be expected to produce less net acid and consequently be less or non-cariogenic. All Strep mutans are non-pH rising except Strep rattus, an arginolytic bacteria which has been associated with a caries free condition in both animals and humans (Kilian et al 1979). According to Kleinberg's mixed- bacterial ecological approach, the ratio of arginolytic to non-arginolytic bacteria is a key factor in the cariogenicity of the Stephan curve. A higher ratio is correlated with a less cariogenic Stephan pH curve whereas with the reverse a more highly cariogenic type of curve. A deficiency in base formation due to lack of saliva or a lower ratio of arginolytic to non-arginolytic bacteria can have similar cariogenic effects as an increased acid formation from a diet high in carbohydrates or a greater ratio of arginolytic to non-arginolytic bacteria.

IV. Focus on Mutans Streptococci and its Virulence Factors

MS possesses certain properties that enable them to predominate in dental plaque. The characteristics are: extracellular polysaccharide synthesis, acidogenicity (acid production), aciduricity (ability to survive in an acidic environment), intracellular polysaccharide (ICP) synthesis and endodextranase production. ICP is produced by some MS in the presence of carbohydrates. When carbohydrate is depleted, ICP can be metabolized leading to continued acid production. Studies of mutant Strep mutans which do not have the ability to produce ICP, while able to colonize teeth of rats, are relatively less cariogenic then non-mutant strains. Strep mutans is a key contributor to the polysaccharide rich extracellular matrix due to the production of glucans by three Gtfs: Gtf B synthesizes mainly insoluble glucans with alpha (1-3) glycosidic linkages, Gtf C synthesizes a mixture of insoluble and soluble glucans and Gtf D synthesizes predominantly soluble glucans with alpha (1-6) linkages. Gtf B and Gtf C are associated with the initial microbial adherence and structural stability of the extracellular matrix. GtfC has the greatest affinity for the hydroxyapatite surface, followed by Gtf B and Gtf D. (Koo 2010) A Gtf-targeted inhibitor can disrupt the formation of capsular-like glucans which make up the biofilm. Since this extra polysaccharide glucan matrix protects the microorganisms from antimicrobial agents, inhibiting glucan synthesis will allow substances such as fluoride or chlorhexidine to better penetrate the biofilm and enhance their biological efficacy. Another advantage of a Gtf-inhibitor is its ability to affect biofilm formation without directly killing bacterial cells. Poor biofilm formation is due to reduced adherence rather than cell death (Ren 2015). In this recent study, the author identified novel molecules that target Gtfs in order to inhibit Strep mutans biofilm formation. Structural based screening of approximately 150,000 commercially available

compounds was performed against the crystal structure of the substrate binding domain of the GtfC protein from Strep mutans. This resulted in the identification of a quinoxaline derivative 2-(4-methoxyphenyl)-N-(3{[2-(4-methoxyphenyl)ethyl]imino}-1,4-dihydro-2-quinoxalinylidene) ethanamine as a potential Gtf inhibitor. This study suggests that a reduction in biofilm formation may be a better strategy to control caries formation as it does not disturb the remaining oral microbiota.

A study by Koo et al 2010 reported on the role of the Strep mutans gtf genes in development of micro colonies and the exopolysaccharide biofilm matrix. Mutants defective in gtfB and C genes showed a loss of ability to synthesize exopolysaccharide matrices, adversely affecting the development of micro colonies. GtfB and Gtfc also produce glucans which enhance the ability of Strep mutans to persist on apatitic surfaces and out compete other oral species. It has been suggested that micro colony development may be linked to bacterial virulence, therefore, this glucan-dependent mechanism has a significant effect on the survival of Strep mutans in complex biofilms. Development of the exopolysaccharide matrix and subsequent acidic niches has significant biological importance, creating shifts in the microbial population. As the environment becomes more acidic, Strep mutans have the ability to upregulate several stress survival pathways. GtfB, GtfC and glucan-binding proteins are further induced, leading to increased biofilm accumulation and colonization of other cariogenic species, such as Lactobacilli. This new environment provides a competitive advantage for all types of aciduric and acidogenic microflora. Consequently, these niches protected by a

diffusion-limiting polymeric matrix results in a biofilm that is recalcitrant to antimicrobials and a challenge to disrupt. (Koo et al 2013)

Another study by Li et al 2010 showed that when Strep mutans were added to saliva biofilms that were allowed to grow for 16 hours, there was a 9 +/-0.4 (SD) fold reduction in Strep mutans incorporated into the biofilms as compared to Strep mutans inoculated with other bacteria and allowing the biofilm to form. This suggests that there is a protective colonization effect of a biofilm prior to addition of Strep mutans.

V. Current and Prospective Therapeutic Approaches Against Dental Caries

A. CHEMICAL MODALITIES - FLUORIDE, CHLORHEXIDINE

Despite fluoride being the most effective anti-caries agent today (Clarkson, 2000), dental caries remains a public health problem among underprivileged populations around the world. Numerous studies have investigated the effects of fluoride and chlorhexidine, both routinely used in clinical practice today, on caries development. Although fluoride's effect on remineralization is well known, it also has important effects on bacteria in dental plaque (Marquis 1995). Fluoride acts directly to inhibit glycolytic enzyme enolase. Fluoride also enhances membrane permeability to protons and compromises the functioning F-ATPases in exporting protons. This induces cytoplasmic acidification and acid inhibition of glycolytic enzymes. (Marquis 1995). Fluoride reduces the acid tolerance of bacteria and is most effective at acidic pH values. Fluoride can prohibit glycolysis in Streptococcus mutans in acidic conditions. The anticaries effect of fluoride not only consists of remineralization but also inhibition of acidogenic bacteria in acidic conditions.

In an in vitro study by Savas 2015, the potency of 5 different substances, 1) 38% Silver Diamine Fluoride 2) Acidulated phosphate fluoride (APF) 3) Ammonium hexafluorosilicate (AHF) 4) Ammonium hexafluorsilicate + cetylpyridinium chloride (AHF + CPC) and 5) 0.2% chlorhexidine - were tested in reducing the number of viable microorganisms in biofilms. The number of viable bacteria was significantly reduced by all of the antibacterial agents. However, silver diamine fluoride (SDF) showed the highest antibacterial activity, with no viable microorganisms detected, while the

effectiveness of the other agents were lower. Another study also reported that after treatment of mature Strep mutans biofilms with SDF, the bacterial count decreased to zero (Chu et al 2012) SDF contains high concentration of silver (253,870ppm) and fluoride (44,800 ppm) ions. Silver ions are bactericidal metal cations that inhibit biofilm formation by inactivating and interfering with bacterial synthesis of cellular polysaccharides through inactivation of the glucosyltransferase enzymes responsible for the synthesis of soluble and insoluble glucans.

In a more recent study, a newly developed nano silver product was tested and evaluated for its antimicrobial and cytotoxicity activity against Strep. mutans. This product, known as nano silver fluoride (NSF), is a combination of silver nanoparticles and chitosan (Santos Jr VE 2014). From previous studies, it was noted that the antibacterial activity of silver nanoparticles increases as the particle size decreases. Chitosan is a biocompatible, biodegradable and non-toxic biopolymer obtained by the deacetylation of chitin. It has antibacterial activity against a broad spectrum of bacteria, including Strep mutans and Strep sanguinis (Targino 2014). The results from this study revealed that NSF may be effective against Strep mutans at much lower doses, does not stain teeth and may have lower toxicity than SDF. The biological effects and mechanism of action of fluoride has been well documented in a paper by Koo 2008. The paper discussed 2 major effects of fluoride on microbial cells: 1) direct inhibition of enzymes in cells, either directly of in the form of metal complexes (Marquis et al 2003) and 2) increasing proton permeability and acting as a transmembrane proton carrier. Fluoride inhibits the release of protons via F-ATPases by moving protons back into the

cell via HF. The cell is 10^7 times more permeable to HF then to F- (Marquis 2003). In the cytoplasm, HF dissociates to H+ and F- and the increased acidity inhibits glycolytic enzymes. Most chemotherapeutic strategies to enhance the biological activity of fluoride are based on the use of broad-spectrum antimicrobials, such as chlorhexidine, triclosan and metal ions/cations which also suppress resident flora (Koo 2008)

Several studies by Koo et al (2002, 2003, 2005) reported on strategies to enhance the biological effects of fluoride on dental biofilms. Apigenin and tt-farnesol, two naturally occurring compounds, have demonstrated anti-cariogenic activity against Strep. mutans in vitro and in vivo. Apigenin inhibits GTF-B and GTF-C and affects the expression of gtfB and gtfC genes, which are responsible for the synthesis of insoluble glucans. tt-farnesol disrupts the proton permeability of Strep. mutans membrane, inhibiting acid production and glucan synthesis by Strep mutans (Koo et al 2003, 2005). Topical application of apigenin and tt-farnesol reduced the incidence of dental caries with minimal effects on the viability of resident microflora in rats (Koo et al 2003). The combination of 1mM apigenin and 5mM tt-farnesol with 250-ppm fluoride was the most effective in reducing the biomass, polysaccharide content and acidogenicity of biofilms (Koo et al 2005).

Chlorhexidine (CHX) is still the most frequently used antimicrobial for reduction of plaque for caries control due to its broad antimicrobial spectrum. However, chlorhexidine rinse has been approved for use to reduce gingivitis but not for caries prevention (Vollmer 2010). A study conducted in 2000 using a 10% chlorhexidine

varnish on adults with low salivary flow rates suggested a significant difference in varnish and placebo treatments on root caries (41% reduction) and total caries increment (25% reduction) (Banting 2000) In the study by Savas 2015, a single application of CHX was found to have significantly lower bactericidal activity compared to other agents such as SDF. Another study reported that a five-minute application of 0.2% CHX did not significantly reduce the viability of microorganisms, however, a 60 minute treatment resulted in a marked reduction in bacterial viability (Pratten et al 1998). Another study reported that 0.2% CHX had an effect in inhibiting Strep mutans in 6 hr biofilms, however its effect was limited to the outer layer of 48 hr dental plaque (Zaura-Arite et al 2001). Similarly, another study reported the application of 0.1% CHX for 1 or 5 min did not have a sufficient effect on 24 hr oral biofilm (Vitkov et al 2005).

B. VACCINES

Early studies of dental caries performed in rats employed whole cells of strep mutans as a possible vaccine (McGhee et al 1975). Later studies focused on vaccines manufactured from various cell-surface antigens of strep mutans and their recombinant fragments or synthetic peptides (Koga et al 1995, Kuramitsu, 1993). Animate (attenuated bacterial factors) and inanimate (liposomes, microparticles) delivery systems have been shown to provide efficient targeting of the vaccine. Both passive and active immunization approaches in animal models and human clinical trials have showed success in delaying the recolonization of Strep mutans.

Molecular targets that have been studied in dental vaccines consists of: 1) blocking receptors necessary for colonization (ex. Adhesins), 2) blocking receptors necessary for accumulation (ex. glucan-binding domains of GBPs and GTFs), 3) inactivating GTF enzymes responsible for glucan formation and 4) enhancing antimicrobial activity of salivary IgA or redirecting synergism with innate components of immunity such as mucin or lactoferrin. Strep mutans adhesins have been identified as antigen I/II, PAc or P1 and are responsible for initial attachment of bacteria to tooth surfaces. There has been abundant in vivo and in vitro evidence to indicate that antibodies with specificity for Strep mutans PAc can interfere with bacterial adherence. PAc in different forms, as a full-length protein, recombinant or synthetic peptide (Smith 2005), protein-carbohydrate conjugate (Wachsmann et al 1986) or DNA based active vaccines (Xu 2007), has been tested in experimental systems and proven as an effective immunogen. Glucosyltransferases produce soluble and insoluble glucans from

sucrose. Antibodies directed to native GTF or sequences associated with its catalytic or glucan-binding function disrupts the synthetic activity of the enzyme and consequently inhibit in vitro plaque formation (Smith et al 1978). Glucan binding proteins (GBPs) bind alpha-1,6-glucan and provide receptors for glucan-mediated aggregation in biofilm development. A study conducted in 1996 by Smith and Taubman in rats injected GBP59, glucosyltransferase (GTF) or phosphate buffered saline (sham) twice at 9-day intervals subcutaneously in the salivary gland vicinity with, with an adjuvant. Two weeks after the second injection, GBP59 and GTF injected rats contained significant levels of salivary IgA and serum IgG to the respective injected antigens. Rats were then infected orally with Strep mutans. Following 71 days of infection, GBP and GTF-injected groups had fewer Strep mutans on their molar surfaces compared with sham-injected infected groups. This suggests that immunization with Strep mutans GBP59 and GTF in rats induces an immune response upon future exposure to cariogenic streptococcus.

Active immunization and passive immunization against dental caries have been extensively documented in the literature. Many active immunization studies demonstrate the important anti-caries role of salivary IgA (Russell et al 1999). Salivary IgA is essentially absent at birth but the oral immune environment undergoes rapid development early in life. Mature salivary IgA is the principal salivary immunoglobulin secreted by individuals at 1 month of age, in addition to considerable concentrations of IgM. By 6-9 months, most children exhibit a more adult-like distribution of salivary IgA1 and IgA2 subclasses (Smith et al 2002). By the second or third year of life, immune responses to streptococcal components such as antigen I/II, glucosyltransferase and

glucan-binding proteins are present and play a role in the ultimate colonization and accumulation of Strep mutans. Several studies have shown that mucosal exposure of humans to gluocosyltransferases of Strep mutans or Strep sobrinus can lead to formation of salivary IgA antibodies (Smith 2002). Five of 7 adults who were orally immunized using enteric coated capsules filled with Strep mutans GS-5 GTF antigen preparations contained in liposomes, had increased levels of parotid salivary IgA antibodies, primarily of the IgA2 subclass (Childers 1994).

Mucosal immunization with GTF could also influence the emergence of Strep mutans in young adults after dental prophylaxis. Young adults were given S. sobrinus GTF administered together with aluminum phosphate topically to the lower lip daily for 5 days. Post dental prophylaxis, there was a delay in re-accumulation of indigenous oral mutans strep, compared with the placebo group given buffer-filled capsules. (Smith and Taubman 1987, 1990). Young adults between 18-42 years of age were screened for levels of antibody activity to GTF in parotid and labial gland salivas and levels of Strep mutans in their whole saliva. Prior to antigen administration, both active and placebo group had similar distributions of Strep mutans in their whole saliva. Although there was no statistical significant difference in anti-GTF IgA antibody activity in parotid or labial salivas between GTF-administered and placebo groups during the 6 weeks following topical application, the proportion of indigenous Strep mutans/total strep flora or total cultivable flora were always lower in whole salivas of the subjects immunized with GTF, compared with placebo. Delays in reaccumulation of microflora was significantly associated with elevations in parotid saliva IgA levels. (Smith and Taubman 1987, 1990)

Passive immunization was tested in earlier studies using mouth rinses containing bovine milk (Filler et al 1991) or hen egg yolk IgY antibody (Hatta et al 1997) to Strep mutans. These studies led to modest short-term decreases in the numbers of indigenous strep in saliva or dental plaque. The effectiveness of a mouth rinse containing antibodies to Strep mutans in preventing the establishment of the bacteria in dental plaque was tested. Immune IgY inhibited Strep mutans adherence to saliva-coated hydroxyapatite discs by 59.2%, while control IgY caused an inhibition of only 8.2%. In the short-term (4-hour) test using a mouth rinse containing 10% sucrose, immune IgY decreased the ratio of the percentage of Strep mutans per total streptococci in saliva. with regards to the ratios of the percentage of Strep mutans per total streptococci in plaque of individual subjects, there was a tendency for a reduction of the ratios in the volunteers receiving the mouth rinse containing immune IgY. These results support the effectiveness of IgY with specificity to Strep mutans grown in the presence of sucrose as an efficient method to control the colonization of mutans streptococci in the oral cavity of humans.

A study by Ma et al in 1990 examined the long-term effects on indigenous microflora after topical application of mouse monoclonal IgG or transgenic plant secretory Secretory IgA/G antibody, with specificity for AgI/II. Subjects' teeth were treated for 9 days with chlorhexidine, then antibody was topically applied for 3 weeks. The group treated with mouse monoclonal IgG did not experience recolonization with mutans for at least 2 years after treatment and the group treated with transgenic

antibody to the Ag I/II epitope did not experience recolonization for at least 4 months. Subjects who were treated with non-specific monoclonals had strep mutans recolonization within 82 days. No clinical side effects were reported in any of the subjects that received monoclonal antibody and there was no evidence of serum, salivary or gingival fluid antibody responses against the antibody. Other studies on experimental passive immunization demonstrated that protection could also be achieved with antibody to GTF (Hamada 1991) or GBP (Smith 2001).

Immunization via intranasal and oral routes have also been reported in previous studies. Mucosal application is generally preferred for induction of salivary IgA since mucosal IgA immune responses are most efficiently induced by administration of vaccines onto mucosal surfaces. The oral route is not ideal because the acidic stomach environment can diminish the effective antigen stimulus before uptake in the GALT (gut associated lymphoid tissue). Intranasal vaccination elicits both humoral and cell mediated antigen-specific immune responses and also requires a much smaller dose of antigen than oral vaccination for induction of antigen-specific mucosal and systemic immune responses. However, in order to develop an effective nasal associated lymphoid tissue (NALT) targeted vaccine delivery system, an adjuvant is necessary. A study that included salmonella-derived protein at the time of pGJA-P/VAX administration resulted in an enhanced IgA and serum IgG antibody response (Shi et al 2012). Flagellum is a ligand for Toll-Like Receptor 5 and is an attractive adjuvant because of its plasticity for generation of fusion proteins of recombinant vaccine antigens (Hayashi et al 2001). TLRs are membrane bound receptors that recognize

pathogen-associated molecular patterns (PAMPs) of invading microorganisms. Binding of TLRs to PAMPs triggers a series of events that activate the immune response.

C. ANTIMICROBIAL PEPTIDES

Between 1920 and 1950, several compounds with antimicrobial properties were isolated from secretions of living beings, and these compounds showed biological activity in small concentrations, with selectivity for both gram positive and negative bacteria. (Rocha da Silva 2012).

One of these compounds was known as Lactoferrin. Lactoferrin is an iron-binding protein found in the innate immune system of mammals biological fluids. It is known to exert broad-spectrum antimicrobial activity against bacteria, fungi, protozoa and viruses. Lactoferrin is known to penetrate the biofilm matrix and directly interact with cell membranes (Ammons et al 2009). Though the mechanism of lactoferrin in biofilm formation has yet to be fully elucidated, some research has attributed its antimicrobial activity to its iron chelating nature. Iron ions participate in a large number of biological processes in microorganisms and are essential in biofilm formation (Weinberg 2004).

Another group of compounds are known as cathelicidins. The cathelicidin proteins have highly conserved N-terminal cathelin domain regions and a C-terminal region that is less well conserved and carries antimicrobial properties. (Rocha da Silva 2012) Only one cathelicidin has been identified in humans, derived proteolytically from the C-terminal end of the hCAP 18 protein. hCAP 18 is expressed by neutrophils and epithelial tissues in the oral cavity and respiratory tract. hCAP18 is expressed and cleaved by proteases to generate a 37 amino acid antimicrobial peptide known as LL-

37. LL-37 has potent antimicrobial activity against Strep species and plays a role in chemotaxis and stimulation of monocytes, T cells, neutrophils and mast cells.

The histatins is another group of antimicrobial cationic proteins produced by the parotid, submandibular and sublingual glands. It exhibits apoptotic activities on microbes and fungi. Histatin-5 is a peptide composed of 24 amino acids and inhibits C. albicans. It binds to receptors on cell membrane and after internalization, inhibits mitochondrial respiration by forming reactive oxygen species which damages the mitochondrial and cytoplasmic membranes, leading to ATP efflux and cell death (Li et al 2006).

Lastly, defensins are composed of more than 380 members, and each contains conserved motifs of 6 cysteine residues forming intramolecular disulfide bonds and beta sheets. They are expressed in response to micro-organism induced stress and play a role in the migration and activation of the innate immune system. (Diamond et al 2009) There are 2 types of defensins: alpha and beta, which are structurally similar but have distinct activities and production sites. Defensins cause cell rupture and inhibit lipopolysaccharide production in oral streptococci (Gomes et al 2010, Schmidt et al 2011). The effect of beta-defensin 3 on 3 week old polymicrobial mature biofilm comprising of oral bacterial species A. naeslundii, L. salivarius, Strep mutans and E.Faecalis was tested using confocal microscopy and dead/live fluorescent staining (Lee et al 2013) Results from the study showed that 24 hour incubation of the biofilm

with hBD-3 (50ug/mL) resulted in a higher percentage of dead cells compared with both untreated samples and samples treated with common disinfectant solutions.

As discussed in earlier section on biofilms, targeting specific bacteria in this complex polymicrobial community is particularly challenging and may result in antibiotic resistance. Conventional treatment of infectious diseases with antibiotics results in killing of most members of the biofilm community, both pathogens and commensal bacteria (Frias-Lopez 2015). Broad killing of bacteria allows for equal competition between Strep mutans and nonpathogenic organisms to re-establish in the biofilm (Sullivan et al 2011). There is an increasing need to develop a specific antimicrobial therapy that can eliminate Strep mutans while leaving the remaining microorganisms in the oral biome intact.

Within the past decade, antimicrobial peptides (AMPs) have been receiving increased attention as potential therapeutic agents because they represent a novel class of antibiotics with a wide spectrum of activity and low rate of inducing bacterial resistance (Di Luca 2014). The number of articles on AMPs and biofilm had reached more than 60 in 2012 and more than 70 in 2013, whereas only a few (<5) articles were published in 2002-2003 (ISI web of science).

Antimicrobial peptides are active against a wide range of microorganisms and metabolically inactive cells. Their main mechanism of action includes permeabilization of the cellular membrane. This is the reason AMPs have very low bacterial resistant

rates as compared to common antibiotics. Emergence of resistance to bilayer-disruptive AMPs would entail changing membrane composition and organization, a “costly” process in evolutionary terms (Zasloff 2002). Since the risk to select resistant strains in biofilms is higher, AMPs are particularly suitable to treat biofilms because of the low rate of induced resistance and efficacy against a wide range of microorganisms.

AMPs are attracted to the surface of microorganisms by electrostatic interactions between anionic or cationic peptide and structures on the cell surface (Lee et al 2011). Several modeling studies have explored the mechanism of action of these antimicrobial peptides on cell death. After binding to the cell surface, AMPs initiate the attachment phase. In this phase, AMPs cross through the outer lipopolysaccharide membrane in gram-negative bacteria or the lipotechoic acid in gram positive bacteria. (Brogden 2005) There are three different models of membrane damage created by antimicrobial peptides. The first is the barrel-stave model where antimicrobial peptides in the membrane form a “barrel”, allowing flow of intracellular contents out into the extracellular environment. The second is the carpet model where AMPs accumulate parallel to the cell surface, oriented to penetrate the lipid bilayer and form pores for the entry of more peptides into the cell. Accumulation of peptides in the cell induces the formation of mycelia which are released from the membrane and leads to its disintegration. The third is the toroidal pore model where AMP helices are inserted into the cell membrane, leading to a connection of opposing lipid monolayers to each other. (Da Silva 2012)

More recently, the discovery of Specifically Targeted Anti-Microbial Peptides (STAMP) has led to a more targeted approach to control oral microbial pathogenesis. A typical STAMP molecule consists of 2 functionally independent moieties conjoined in a linear peptide sequent - 1) a non-specific antimicrobial peptide serving as the killing moiety and 2) a species-specific binding peptide serving as the targeting moiety and provides specific binding to a selected pathogen and facilitates the targeted delivery of the antimicrobial peptide. A STAMP have been shown to be effective in eliminating Strep mutans from a mixed-species environment without affecting closely related non-cariogenic oral streptococci (Eckert et al 2006).

In a study by Eckert, a pheromone produced by Strep mutans, known as the competence stimulating peptide (CSP) is used as the targeting domain for Strep mutans- specific delivery of the antimicrobial. The initial CSP-derived STAMP was constructed by synthesizing full-length Strep mutans CSP (21 amino acids) with the antimicrobial peptide G2 (16 amino acids derived from the antimicrobial peptide novispirin G10). This initial AMP did not reveal any antimicrobial activity possibly due to steric hindrance. Therefore, a shorter targeting domain (16 amino acids) was created. C16G2 consists of a Strep mutans-selective pheromone “targeting region” comprising a fragment of CSP (C16) conjoined to a novispirin-derived “killing region” consisting of a broad-spectrum antimicrobial peptide (G2). The C16G2 rinse was associated with reductions in plaque and salivary Strep mutans, lactic acid production and enamel demineralization. However, the impact on total plaque bacteria was minimal. Short exposure of C16G2 was capable of selectively inhibiting the growth of Strep mutans

within a multi species biofilm and in the presence of saliva for a minimum of 2 hours without disrupting the overall health of the biofilm (Hinkle 2013). C16G2 has a rapid mechanism of action working within less than 1 minute of exposure to bacteria. The half-life of C16G2 however was 18.8 minutes, suggesting it is unlikely to have meaningful effect in the oral cavity after long durations. The C16G2 rinse was also shown to significantly elevate resting pH of dental plaque in comparison to placebo rinse.

A study by Sullivan et al 2011 reported on the clinical efficacy of STAMP mouth rinse. The authors reaffirmed the need to develop a specific antimicrobial therapy to eliminate the primary cause of dental caries, Strep mutans from the oral biome without disrupting the remaining microflora. Broad killing of the bacteria allows for equal competition between Strep mutans and non-pathogenic organisms to re-establish themselves in the biofilm (Sullivan 2011) While previous studies by Eckert et al 2006 has established efficacy and specificity against Strep mutans in biofilm communities and planktonic cultures, it was unclear whether C16G2 remained active in vitro as a mouth rinse. A 40s mouth rinse containing 0.04% C16G2 was administered once at the start of a 4-day test phase, and no fluoride tooth paste was used. Results showed that C16G2 in 1xPBS (Phosphate Buffered Saline) reduced the viability of Strep mutans to below 10% of mock-treated controls. This suggest that C16G2 was effective against Strep mutans at therapeutic concentrations of 25-100uM. Furthermore, in vitro hemolysis and tissue irritation assays were performed against human cells. C16G2 had no hemolytic activity against human RBCs at concentrations 100uM or less. Additional cytotoxicity

test using fully differentiated buccal (EpiOral) and gingival (EpiGingival) tissues from healthy donors were cultivated in-vitro and exposed to C16G2 (25 or 100uM). Results showed that no ET-50 (time to reduce tissue viability to 50%) could be obtained for STAMP-treated EpiOral and EpiGingival. To determine if the selective killing of Strep. mutans translated into a decrease in enamel demineralization, an intra-oral caries model was used. Enamel specimens were mounted in an upper palatal retainer and exposed to 10% sucrose challenges 4x daily. The results showed a 3.73% increase in enamel hardness relative to baseline in the C16G2 group, whereas a 23.0% loss of enamel hardness in the placebo rinse group. C16G2 was also shown to significantly elevate the resting pH of dental plaque relative to the placebo rinse (7.14 vs 6.66). This increase in pH has 2 benefits: Firstly, a higher resting pH promotes faster remineralization and decreases the severity of subsequent acid challenges. Secondly, higher resting pH encourages the growth of healthy bacteria and is unfavorable for cariogenic bacteria.

In another study by Wang et al 2015, a short synthetic amphiphilic peptide known as 1018 was effective in inhibiting biofilm development and killing of organisms in the biofilm. This anti-biofilm peptide binds to and stimulates the degradation of second messenger nucleotide (p)ppGpp, involved in biofilm formation and maintenance. 10ug/ml of peptide 1018 (below MIC of >80ug/ml) had successfully inhibited plaque biofilm formation by suppressing more than 75% of biofilm growth, compared to the water control group. Peptide 1018 was not degraded by salivary mucins, unlike LL-37 which had a decreased antimicrobial activity due to saliva (Bucki et al 2008). The study

also looked at the effect of peptide 1018 in combination with CHX to treat established plaque biofilms. While the combination of 1018 and CHX did not significantly reduce biofilm volume compared with each agent when used alone, the combined treatment led to significant increase in the amount of dead cells within the biofilms.

There have been several issues raised in previous studies on AMPs. Although the development of resistance to AMPs is slower than antibiotics, it is well established that bacteria adopt a variety of efficient strategies to resist even antimicrobial peptides. Some of the bacterial cationic antimicrobial peptides resistance mechanisms include electrostatic repulsion of CAMPs by modification of cell envelope molecules, proteolytic cleavage of CAMPs, production of CAMP-trapping proteins or extrusion of CAMPs by energy-dependent efflux pumps. Others have reported resistance to antimicrobial peptides is mainly based on the interaction with biofilm and capsule exopolymers. These polymers work by electrostatic repulsion and/or sequestration of these antibacterial substances (Kraus & Peschel 2006, Otto 2006). Other limitations to AMPs include potential toxicity, susceptibility to proteases and the high cost of peptide production. Designing the appropriate drug delivery system for AMPs is challenging because of enzymatic degradation and unfavorable physiochemical properties such as molecular aggregation and serum sequestration (Di Luca 2014). The high cationic activity of most AMPs in non-physiological conditions is usually significantly reduced in biological fluids such as plasma, serum or silver. It is also difficult to use parenterally because of high toxicity and quick renal excretion. (da Silva 2012)

Current strategies under investigation include chemical modification, formulation vehicles and co-treatment with enzyme inhibitors or absorption enhancers. The use of peptido-mimetics is a strategy to avoid proteolytic degradation and the design of short peptides retaining antimicrobial activities is a solution to decreasing cost (Seo 2012). A study proposed that there was a protective colonization effect of biofilm from Strep mutans. They hypothesized that targeted elimination of Strep mutans leads to the generation of protective biofilms with the ability to prevent secondary surface colonization by Strep mutans (Li et al 2010). After 16 hours of inoculation of STAMPs with spectinomycin-resistant Strep mutans derivative JM11, there was no detectable Strep mutans cells in the sample while the untreated control contained ~10^7 Strep mutans cells in the biofilm. The efficacy of STAMP on shorter exposure duration times was also tested and results showed that biofilms treated for as little as five minutes contained approximately four (STAMPE 2_1G2) and five -(STAMP C16G2) orders of magnitude less Strep mutans cells. Furthermore, re-infection with Strep mutans was 38+/- 13 (2_1G2) to 61 +/-13 fold (C16G2) significantly less in treated biofilms compared to untreated controls. The outcome of this study provided evidence for the protective effect of a “normal” oral biofilm similar to the concept of “window of infection” proposed by Caulfield et al 1993. In addition, the results suggests that whether naturally or through STAMP treatment, established Strep mutans-free biofilm reduces the competitive advantage of Strep mutans even in the presence of high sugar content, preventing the shift in biofilm composition toward cariogenesis (Li et al 2010).

D. REPLACEMENT THERAPY

Several studies on replacement therapy were published in the early twenty-first century by the Hillman group. The basis of replacement therapy is the implantation of relatively innocuous “effector” bacteria that can competitively exclude or outgrow potentially disease-causing bacteria, without significantly disrupting the balance of the existing microbial system. Hillman identified 4 properties of an effective genetically modified effector strain for use in replacement therapy of dental caries. Firstly, it must have a significantly reduced pathogenic potential. Secondly, it must persistently and preemptively colonize the Strep mutans niche, preventing colonization by wild-type strains whenever the host comes into contact with them. Thirdly, it should be able to aggressively displace indigenous strains of Strep mutans, allowing even previously infected subjects to be treated with replacement therapy. Lastly, it must be generally safe and not predispose the host to other disease conditions (Hillman 2000).

They explored techniques for reducing cariogenicity in effector strains. Initially, mutants which had defects in glucan synthesis demonstrated reduced cariogenicity in animal models. However, they were unlikely to successfully outcompete glucan-synthesizing strains for prime biofilm niches. Therefore, attention shifted toward introducing effector strains with mutations affecting acid production. These were called lactate dehydrogenase mutants (LDH). Lactate dehydrogenase mutants produced less lactic acid and had reduced cariogenicity in rodents, however, this mutation was lethal

in Strep mutans. A zygomonas mobilis gene encoding alcohol dehydrogenase was then inserted, also known as BCS3-L1 with virtual deletion of all the LDH gene. This created a strain that had traits of low acid production and strong mutacin production. BCS3-L1 yielded final pH values that were 0.4-1.2 pH units higher than those of its parent. The reduced acidogenic potential is a major factor in its reduced cariogenic potential (Hillman 2000)

BCS3-L1 was created from a clinical Strep mutans isolate. Recombinant DNA technology was used to delete the gene encoding lactate dehydrogenase entirely and also designed to produce elevated amounts of novel peptide antibiotic known as mutacin 1140 (Hillman 2002), that gives it a strong selective advantage over most other strains of Strep mutans. Mutacin production was tested in a rat model and shown to be a phenotypic property that allows the bacteria to preemptively colonize and aggressively displace other bacteria. This was tested in a study by Hillman et al 1998. 3 years following a single, 3 minute infection regimen involving brushing and flossing, a concentrated cell suspension placed onto and between the teeth resulted in all human subjects remaining colonized by the mutant strain and producing a 3 fold elevated amount of mutacin 1140. Reacquisition of an acidogenic phenotype by spontaneous reversion is also extremely unlikely because of the deletion of the entire ldh gene in the construction of BCS3-L1.

One of the greatest advantages of replacement therapy is no need for patient compliance. However, there are several disadvantages with replacement therapy.

Firstly, even low-virulence colonizing strain can cause infections in immune-compromised individuals. Secondly, the high intrinsic stability of the indigenous bacteria in the biofilm can present a major obstacle in introducing a new effector strain. Success is highly unlikely unless the effector strain is competitive (Hillman 2002). Alternatively, the effector strain could be introduced either before establishment of the microbial community, for instance in children immediately after the onset of tooth eruption and before their acquisition of a wild-type strain or upon creation of an appropriate niche following disruption of microflora by exposure to antimicrobials (Tagg 2003). Numerous early studies have reported on the difficulty in persistently introducing laboratory strains of Strep mutans into the mouth of humans, particularly after the indigenous microbiota has been established. (Krasse et al 1967l Jordan et al 1972; Ruangsri et al 1977; Svanberg et al 1981)

While creation of an innocuous effector strain has its challenges, studies have reported on other strains of Streptococci which can competitively inhibit caries-causing Strep mutans. A study by Becker et al in 2002 found that healthy subjects had significantly higher number of S. sanguinis, whereas subjects with caries possessed almost no detectable levels of S. sanguinis. High levels of S. sanguinis was correlated with delayed acquisition of Strep mutans. (Caulfield et al 2000).

It has been documented that Strep sanguinis, Strep gordonii and Strep mutans compete within the same niche. (Kreth et al, 2005). The success of colonization is determined by the sequence of establishment, nutritional availability and environmental

pressures. Strep mutans produce bacteriocins which can inhibit the growth of other streptococci. However, Strep gordinii and Strep sanguinis are able to inhibit the growth of Strep mutans by producing hydrogen peroxide (H2O2). Bacterocin production via the ComDE- QS system usually occurs with an increased amount of CSP when cell density is high. The production of H2O2 therefore, might provide an ecological advantage over bacteriocin production during the initial colonization, when cell density is not high enough to trigger bacteriocin production. H2O2 production is dependent on oxygen and usually occurs during initial colonization (Marquis 1995). Oxygen tension decreases after the biofilm reaches a certain thickness and cell density due to diffusion limitations. Subsequently under these conditions, Strep mutans has the competitive advantage producing bacteriocins to inhibit Strep gordonii and Strep sanguinis.

Wang and Kuramitsu (2005) reported that suppressants from Strep gordonii are able to inhibit the production of bacteriocins, but not affect the bacteriocins directly. They identified a protease called challisin, which is able to degrade CSP, and thus disrupt the bacteriocin/competence regulation cascade. A search of the Strep sanguinis genome also reveals a homolog of challisin. Another competitive inhibitor was identified as Strep oligofermentans. Strep oligofermentans is able to utilize lactic acid produced by cariogenic species such as Strep mutans to generate H2O2, consequently inhibiting Strep mutans.

E. QUORUM SENSING TARGETS

Quorum sensing (QS) is a cell density dependent communication process that respond to the inter/intra-species signals and elicit responses to show behavioral changes in the bacteria to aggressive forms. Bacteria communicate with each other using hormone-like molecules known as pheromones, which increase in concentration as a function of bacterial cell density. (Leung et al 2015)

A major mechanism of signal transduction in bacteria is the so-called two-component signal transduction system (TCTSs) (Li et al 2008), which enable bacteria to regulate their gene expression and coordinate activities in response to environmental stimuli. A typical TCTSs consists of a membrane-associated histidine kinase (HK) protein, which senses a specific stimulus, and a cytoplasmic response regulator (RR) protein, which enables the cells to respond to the stimulus via regulation of gene expression (Hoch, 2000).

In Strep Mutans, the QS system known as CSP-ComDE, is achieved by production and detection of signaling molecules in the form of a small peptide named CSP (competence-stimulating peptide) pheromone. The CSP pheromone accumulates not in proportion to typical cell density, but rather, to particular stresses in the oral cavity. This suggests that the pheromone probably functions as a stress-inducible alarmone, signaling bacteria in the biofilm to initiate an adaptive response that results in different phenotypic outcomes (Leung et al 2015). The CSP pheromone, once reaching

its threshold concentration, directly interacts with the membrane-bound histidine kinase receptor, ComD, to orchestrate a signaling response. The interaction of CSP pheromone with ComD triggers the dimerization and autophosphorylation of the receptor, and initiates the phosphorylation and subsequent activation of cytoplasmic response regulator ComE. Activated ComE directly activates the expression of several genes encoding bacteriocins and bacteriocin-like peptides and indirectly regulates SigX, involved in the control of the competence regulon.

Interference with the QS signaling is a promising avenue toward development of novel therapeutics. QS peptides could be useful for inducing targeting suicide. Alternatively, diminishing QS mechanisms could be considered to prevent persistence and genetic heterogeneity through the spreading of potential antibiotic resistance genes. (Leung 2015) The ability to interfere with bacterial QS could manipulate the composition of pathogenic biofilms. Once the threshold concentration is reached (“quorum” or the number of bacteria required to activate the QS system), the QS molecule initiates a signaling cascade culminating in a population-wide differential regulation of target genes enabling bacteria to act as multicellular organisms. The CSP-dependent QS system in Strep mutans has been found to regulate physiological activities including bacteriocin production, competence development, biofilm formation and stress response.

Bacterial programmed cell death is a kind of altruistic act that provides a way for species to survive stresses at the expense of some of its cells. Death by suicide of a subpopulation may leave more resources for the surviving population and thereby improve long-term survival of the species. Previous studies have shown that CSP-induced PCD was involved in the release of extracellular DNA in the biofilm, "contributing to the architecture of the biofilm matrix and providing a mechanism for dissemination of fitness-enhancing genes under stress" (Leung 2015). In a study by Zhang et al 2009, late-stage biofilm formation by Strep mutans was significantly increased in the presence of exogenous CSP. The addition of synthetic CSP correlated with cell death, release of genomic DNA, induced competence for DNA uptake and increased biofilm formation. Cell death benefits the attachment of surviving cells and induces subsequent biofilm differentiation and dispersal. (Bayles et al 2007, Petersen et al 2005)

Some bacteria enter a state of normalcy. These non-growing dormant cells known as persisters, are tolerant to all antibiotics currently in use without expressing a drug resistance mechanism (Leung 2015). Persisters are phenotypic variants of the wild-type strain that arise in a clonal population of genetically identical cells. Since persisters are in a growth-arrested physiological state, antibiotics that target essential cellular processes are ineffective in killing these cells. The formation of persisters has been described as "spontaneous" (Cohen 2013) and the existence of a small subpopulation of persisters in any growing bacterial population reflects a population-level strategy of survival in a rapidly changing environment. QS-deficient mutants did

not demonstrate the ability to produce persister phenotypes which suggests that the CSP-ComDE QS system may be a deterministic mechanism for persister formation (Leung 2012). Recently, it was shown that an intact signal relay between ComDE, Com RS to the activation of SigX was required for the stress-induced persistence phenotype (Leung et al, 2015). These results showed the importance of the QS system in mounting an adaptive stress response within a subpopulation of S.mutans to form dormant persisters for survival of the species.

Among the therapeutic targets in QS system are CSP. STAMPs (selectively targeted antimicrobial peptides) were designed to have a targeted domain (8-amino acid region of the CSP) fused to an antimicrobial peptide domain and shows a robust species-specific activity that eliminates S.mutans without affecting the other non-cariogenic streptococci. It was reported that C-terminal truncated CSP peptides could competitively affect the QS activity and structural motif in the C-terminal domain restoring the activation of the QS signal transduction pathway (Syvitski 2007). Another therapeutic target is the autoinducer-2 (AI-2). AI-2 is a furanosyl borate diester, known to mediate interspecies communication. AI-2 is encoded by the highly conserved luxS gene, and mutation in the luxS gene of Strep mutans leads to an altered biofilm structure and also decreases the production of bacteriocin and mutacin I (Yoshida et al 2005, Merritt et al 2003). A study showed that synthetic furanone (Z)-5-bromomethylene-2(5H)-furanone, a potential inhibitor of AI-2, was able to reduce biofilm formation in Strep anginosus, Strep intermedius and Strep mutans wild type strains, both when coated on a surface or when added to medium. (Lonn-Stensrud et al 2007)

The effect was more pronounced on furanone-coated surfaces, where the reduction was 76% and 63% in Strep intermedius and Strep mutans respectively. The reduced biofilm formation in all 3 streptococci studied demonstrated that the effect of furanone was not species specific. Furthermore, the furanone concentrations used had no effect on total growth of bacteria and therefore, reduces the risk of antimicrobial resistance development. Another therapeutic target is the ABC transporter (ComA), which play a vital role in the maturation and secretion of CSP (Kotake et al 2008). More specifically, the PEP domains of ComA would be an ideal target for the development of drugs to inhibit biofilm formation. Mutations at the active site of PEP has resulted in the complete loss of the catalytic activity of PEP domain. Based on the observation and substrate specificity of peptidase domain of ComA, Kaur et al 2015 hypothesized that the therapeutic inhibition of the PEP domain of ComA will halt the maturation and secretion of CSP from Strep mutans.

Another QS system known as HK/RR 11 is involved in Strep mutans survival at acidic pH. (Li et al 2008). Simultaneous inactivation of the ComCDE quorum-sensing system and HK/RR11 two-component regulatory system resulted in additive attenuation of the virulence and cariogenic potential of Strep mutans, since inactivation of either of these systems alone did not result in effects of the same degree or extent. This suggests that the ComCDE and HK/RR11 signal transduction systems function independently to regulate physiological activities, ecological fitness and virulence of Strep mutans (Li et al 2008). An interesting finding of this study was that inactivation of the ComCDE QS system alone did not affect oral colonization and succession of Strep

mutans in rats, however the caries score was significantly lower than that of the parental strain, indicating that colonization of Strep mutans in dental biofilms may not be sufficient to explain its virulence and cariogenic activity, although it is a pre-requisite for infection (Li et al 2008). The ComCDE system also controls production of several bacteriocin and bacteriocin immunity proteins. These compounds can kill other related species in favor of Strep mutans in the biofilm, and the release of DNA can be used by Strep mutans for genetic exchange (Kreth et al 2005)

Several recent studies have described the strategy and application of quorum-sensing antagonists to prevent opportunistic infections caused by P. aeruginosa and S. aureus (Hentzer et al 2003). The major advantage in using anti-quorum-sensing compounds is that it specifically blocks or overrides bacterial signaling pathways that confer pathogenicity without significant effects on bacterial viability as a whole. When bacterial viability is not disturbed, there is much less selection pressure to create resistant microbes (Li et al 2008).

F. NANOPARTICLES

Nanoparticles are classified as particles with a size no greater than 100nm. Silver and copper have received the most attention in dentistry. An inverse relationship between the size of nanoparticles and antimicrobial activity has been demonstrated. a range of 1-10nm have been shown to have the greatest killing activity against bacteria compared with larger particles (Morones et al 2005) Bacteria are less likely to acquire resistance to metal nanoparticles compared to conventional broad-spectrum antibiotics because metals act on a broad range of microbial targets and many mutations would have to occur in order for micro-organisms to resist their antimicrobial activity (Allaker et al 2014) Metal oxides which produce a reactive oxygen species when exposed to UV light such as titanium dioxide (TiO2) and since oxide (ZnO) are increasingly used in antimicrobial applications (Allaker et al 2014)

A pertinent issue in restorative and preventative dentistry today involves the development of secondary caries. Traditional composites in vivo have been shown to accumulate more biofilms than other restorative materials. Plaque formation adjacent to restoration margins could result in secondary caries. More than half of all restorations fail within 10 years and 50-70% of all restorations placed are replacements of failed restorations (Cheng et al 2015). There has been a surge in focus on restorative materials which can minimize the risk of secondary caries. A study by Cheng et al 2015 investigated the incorporation of nanoparticles of silver (NAg) into

composites/adhesives and quaternary ammonium methacrylates (QAMs) to combat biofilms. Nanoparticles of amorphous calcium phosphate (NACP) showed release of calcium/phosphate ions which remineralized tooth lesions and neutralized acids. The combination of NAg, QAM and NACP lead to the formation of a new class of composites and adhesives which is not only effective against bacteria but also promotes remineralization.

Silver has been extensively investigated for antimicrobial activity and is also relatively less toxic to human cells at very low concentrations. Silver has antibacterial, antifungal and antiviral properties. Though mechanism of action of Ag+ is not completely understood, studies have shown that the positive charge on Ag+ creates an electrostatic attraction between the negative charge of the bacterial cell membrane. Proteins in membrane or phosphorous containing elements such as DNA may be preferential binding sites for silver nanoparticles (Kim et al 2007) Nano Ag (Nag) has a high surface-area-to-mass ratio, hence a small amount of NAg was sufficient for the composite to be strongly antibacterial. Furthermore, a small amount of NAg did not compromise the color, esthetics or mechanical properties of the composite. (Cheng et al 2015) Silver ions have the following 3 main antibacterial effects: destruction of cell wall structure; denaturation of cytoplasmic enzyme and inhibition of microbic DNA replication (Peng et al 2012).

In a study by Cheng et al. 2012, nano composites were developed containing amorphous calcium phosphate (ACP) or calcium fluoride (CaF2) nanoparticles and CHX

particles. Four nano composites were created with fillers: nano ACP, nano ACP + 10% CHX, nano CaF2, nano CaF2 + 10% CHX. Results showed that the release of CHX from the ACP nano composite was similar to that of the CaF2 nanocomposite. After 3 days, the NanoCaF2 + CHX and NanoACP + CHX had pH levels that remained at 6.5 or higher. For NaCaF2 alone, the pH dropped to 4.6, while NanoACP alone fell to a pH of 4.2. Furthermore, nano composites with CHX decreased CFU Strep. mutan counts by 1000 fold compared to regular non-releasing composite. This study demonstrated that restorations that release CHX have the potential of eliminating bacteria in the vicinity. Consequently, this helps promote recolonization of the area with non-acidogenic bacteria and prevent recurrent caries. NanoCaF2 + CHX had a biofilm pH comparable to NanoACP + CHX, and the author proposed that this is likely due to the F ion release of CaF2. The F ion can help reduce acid production of bacteria, via inhibition of metabolic pathways. In addition to the anti-cariogenic activity of these nanoparticle materials, the flexural strength also remains comparable with commercial composites without fluoride.

Metal oxide nanoparticles have also been studied for its antimicrobial properties. The common oxides are those of copper, zinc, titanium and tungsten. Since metals target a broad range of microbial targets, development of resistance against metal oxide nanoparticles is highly unlikely (Allaker et al 2014). Zinc oxide has been shown to have a high degree of selective toxicity to bacteria and good biocompatibility. In a study by Eshed et al 2012, sonochemical coating of artificial teeth with ZnO and CuO nanoparticles significantly inhibited biofilm formation of Strep mutans. In another

study investigating the antibacterial properties of incoporating silver and zinc oxide nanoparticles in composite resins (Kasraei et al 2014), results showed that composite resins containing silver or zinc-oxide had a higher antibacterial activity against Strep mutans and Lactobacillus. Ramazanzadeh et al 2015 published findings on a study comparing the antibacterial effects of ZnO and CuO nanoparticles coated brackets on Strep mutans. The study found that CuO and ZnO-CuO nanoparticles coated brackets have better antimicrobial effect on Strep mutans than ZnO coated brackets. In fact, no colony growth was seen after 2 hours in both CuO and CuO-ZnO coated brackets. The growth of Strep mutans was significantly reduced by ZnO nanoparticles after 6-24 hours.

VIII. NATURAL PRODUCTS

Natural products have been shown to inhibit bacterial growth, acid production and acidurance, exopolysaccharide synthesis and bacterial adherence. These products are usually associated with secondary metabolites produced by an organism, and there are 3 main groups: 1) phenolic compounds containing benzene rings, hydrogen and oxygen 2) alkaloids - nitrogen-containing compounds and 3) terpenoids - made from mevalonic acid and composed almost entirely of carbon and hydrogen.

Among the inhibitors of bacterial growth, Camellia sinensis has been shown to contain specific catechins from green tea which have antibacterial activity against S. mutants and S. sobrinus. The antibacterial inhibitory effect seems to be related to the "presence of three hydroxy moieties at 3', 4' and 5' on the B ring of the catechin and epicatechin molecular structure". (Sakanaka et al 1989). Essential oils affect the bacterial viability by compromising the integrity of the bacterial membrane. Terpenoids is the main chemical group. Thymol and eugenol inhibit a wide range of oral microorganisms including Strep mutans (Shapiro et al 1994). The antibacterial activity of tt-farnesol, oleic and linoleic acids may be related to their lipophilic characteristics and increase proton permeability.

Inhibitors of acid production and acidurance include 7-epiclusianone, tt-farnesol and some cranberry flavonoids. These have been reported to increase the proton permeability of Strep mutans cells, causing cytoplasmic acidification and inhibiting the acid-sensitive intracellular glycolytic enzymes (Koo et al 2010). Psidium chatelaine and epigallocatechin gullet have been shown to disrupt the expression and activity of

specific enzymes involved in the glycolytic pathway of Strep mutans, including lactate dehydrogenase. (Hirasawa et al 2006, Brighenti et al 2008)

Polyphenolic compounds have been extensively studied in their ability to reduce gluten synthesis and exopolysaccharide synthesis. Various polyphenols from the leaves of C.Sinensis (extracts of green or oolong tea), propolis, cacao bean husk, cranberry and often traditional plants (Serindeia warnecki, Azadirachta indica, R.gardneriana and M. chances) exhibit inhibitory effects against gluten synthesis (Jeon et al 2011). Most of the effective compounds in natural products have been found in polyphenolic compounds (Cheng et al 2015).

IX. CONCLUSION

Dental caries remains an infectious disease and a public health burden worldwide. Almost 95% of the world population is affected by caries at different ages. While usually not life-threatening, the development of cavities is inversely related to quality of life and overall health. (Sullivan 2011) With the introduction of refined sugar into the modern diet and an improved understanding of the pathogenesis of dental caries, the need for novel approaches to caries prevention has become is a key undertaking for addressing the caries burden in the 21st century.

Currently, the most effective measures against caries development is the use of fluoride-containing products, such as varnishes, toothpastes and silver diamine products. Individual behaviors with regard to oral hygiene and diet is also key in the balance between cariogenic and non-cariogenic plaque. (Sullivan et al 2011) The wide-spectrum antibiotic use combined with emergence of drug resistant strains highlight the fundamental need for new “targeted” antibiotic therapies to combat specific mucosal pathogens with minimal impact on the remaining oral microflora (Eckert et al 2012). Furthermore, broad killing of bacteria results in equal competition between Strep mutans and nonpathogenic organisms in the re-establishment of the biofilm.

While fluoride is able to reduce the incidence and prevalence of dental caries and should remain an important component of oral health regimen, it has limited efficacy in killing cariogenic bacteria residing in dental plaque (Hamilton 1990). This could explain

the persistence of dental caries within populations, despite fluoride's well documented clinical efficacy (Milgrom et al 2009, Anderson and Shi 2006).

Vaccines including both animate (attenuated bacterial factors) and inanimate (liposomes, microparticles) delivery systems have been shown to provide efficient targeting of Streptococcus mutans. Both passive and active immunization approaches in animal models and human clinical trials have shown success in delaying the recolonization of Strep mutans. (Russell et al 1999, Ma et al 1990)

Antimicrobial peptides have been receiving increased attention as potential therapeutic agents within the past decade because they represent a novel class of antibiotics with a wide spectrum of activity and low rate of inducing bacterial resistance. Specifically Targeted Antimicrobial Peptides (STAMPs) have been developed comprising of a species-specific targeting region, linker region and a wide-spectrum killing domain. STAMP treatment has received approval by the Food and Drug Administration to be studied as an Investigational New Drug and is currently in Phase II clinical trials for safety and efficacy. (Eckert et al 2006, 2012, Kaplan et al 2011)

Replacement therapy involves the implantation of relatively innocuous "effector" bacteria that can competitively exclude or outgrow potentially disease-causing bacteria, without significantly disrupting the balance of the existing microbial system. Many early studies however, have reported on the challenge in successfully introducing effector strains into the mouth of humans. (Hillman et al 2000, 2002, Tagg et al 2003)

Quorum sensing is the secretion and detection of a diffusible molecule by the community members can lead microbial species to suddenly change their behavior. Interference with the QS signaling is a promising avenue toward development of novel

therapeutics. QS peptides could be useful for inducing bacterial programmed cell death. The ability to interfere with bacterial QS could manipulate the composition of pathogenic biofilms. (Leung et al 2015)

Nanoparticles have become increasingly studied in its use in composite dental materials. This approach was developed with the goal of reducing the incidence of secondary caries. It has been reported that bacteria are less likely to acquire resistance to metal nanoparticles compared to conventional broad-spectrum antibiotics because metals act on a broad range of microbial targets and many mutations would have to occur in order for micro-organisms to resist their antimicrobial activity. (Allaker et al 2014)

Lastly, natural products have been shown in in vitro experiments to inhibit bacterial growth, acid production and acidurance, exopolysaccharide synthesis and bacterial adherence. (Jeon JG 2011) The 3 main groups of natural products include phenolic compounds, alkaloids and terpenoids, of which phenolic compounds have been proven most effective. However, the specific mechanisms of anticaries effects are still unclear for most natural products. (Cheng et al 2015)

While these treatment modalities have been shown to target different virulence factors of Strep mutans, there is not one approach that has consistently shown a safe and efficacious approach against caries in humans. With the goal in mind to address the large caries burden worldwide, the next step would be to develop a new drug that could predictably and selectively target Strep mutans while being readily accessible to all populations regardless of socioeconomic status.

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