PROJECT TITLE

Ecological approaches to dental caries prevention: A controlled trial to evaluate the influence of CPP-ACP – Cranberry toothpastes in effecting an ecological change in the oral plaque microbiome

SHORT TITLE

Natural Products Trial

1. BACKGROUND AND SIGNIFICANCE OF STUDY

1.1 Introduction

Oral health is integral to general well-being, with profound individual and societal implications that extend well beyond the functions of the craniofacial complex. Diseases such as dental caries are major public health concerns, imposing a costly burden on health services. Traditional caries epidemiological measures do not adequately reflect the social impacts, economic costs, and health care system effects of the disease [Casamassimo et al., 2009]. Although largely preventable, dental caries remains the most common chronic disease in children and is a major contributor to tooth loss in adults [Benjamin, 2010]. Dental caries prevention has for long been narrowly focussed on fluoride therapies. However, contemporary disease paradigms emphasise that prevention of dental caries needs to be directed towards countering the biofilm microbial mixtures responsible for acid production as well as correcting the disturbed oral plaque microbial ecology [Walsh, 2011].

1.2 Rationale for ecological caries preventive approaches

The widespread use of fluoride-based caries preventive programs (primarily as fluoride dentifrices), from the latter half of the 20th century, did result in significant improvements in caries prevalence rates of developed countries. However, more recent epidemiological data is showing a worrying trend, with the past improvements seen in caries prevalence rates plateauing and even reversing in some sections of the population [Agustsdottir et al., 2010; Bagramian et al., 2009; Dye et al., 2017]. Two key summaries from the 2015 Australian Institute of Health and Welfare report were that 55% of 6-year olds had experienced decay in their primary teeth, and that 48% of 12-year old children had their permanent teeth affected by dental caries [Chrisopoulos S et al., 2016]. In fact, the most recent Global Burden of Disease report reveals that untreated caries in permanent teeth still remains the most prevalent human disease condition worldwide, with untreated caries in primary teeth being the 10th most prevalent disease [Kassebaum et al., 2015]. The flattening of caries prevalence rates, even in dentally aware populations that commonly brush their teeth with fluoridated dentifrices, is concerning and underscores the need to develop new-age caries preventive strategies that are synergistic with or complementary to fluoride.

Dental caries is today recognised as a behavioural disease that arises due an environment-induced shift in the microbial ecology of the oral plaque biofilm. Environmental factors like frequent dietary exposure to fermentable carbohydrates or salivary dysfunction allow the acidogenic/aciduric members of resident oral flora to obtain a selective ecological advantage over other bacterial species, disrupting the homeostatic balance of the biofilm, and initiating the disease process [Marsh, 2003]. A key principle of this ecological plaque hypothesis is that unless there is an attempt to interfere with the environmental factors driving the biofilm dysbiosis, the patient is likely to suffer from repeated episodes of the disease, and the clinician will encounter frequent failure of any restorative or preventive treatment rendered [Marsh et al., 2015].

Despite these paradigm shifts in the aetiological concepts of the disease, caries prevention remains largely dependent on twice-daily brushing with fluoride dentifrices and advising individuals to restrict their dietary sugar exposures. The cariostatic actions of fluoride are largely attributed to its physiochemical effects while its biological effects on oral plaque biofilms at concentrations found in the oral cavity are limited. The most effective option to avoid a cariogenic oral plaque biofilm is to limit dietary sugar exposures. However, dietary behaviour modification advice is particularly difficult to follow in a society where cariogenic snacks/drinks are easily available. This necessitates the development of alternate ecological caries preventive measures that can beneficially modify oral plaque biofilms and reduce it virulence attributes.

Evolving evidence is also increasingly highlighting the beneficial aspects of a healthy resident oral microbiome [Kilian et al., 2016]. Under 'normal' environmental conditions, the resident oral microbiome has a symbiotic relationship with the host characterised by commensalism and mutualism [Ruby and Goldner, 2007]. Thus, while dental caries is undoubtedly a biofilm-mediated disease, it is imperative not to perturb or lose the beneficial functions delivered by commensal microbes. Consumer oral care products should ideally focus on maintaining the composition and activity of healthy biofilms rather than eliminating them completely. Adopting ecological approaches to dental caries prevention can potentially preserve the favourable effects that the host derives from the resident oral microbiome, while reducing cariogenic virulence factors that are responsible for the oral plaque biofilm dysbiosis. Plaque modifying agents that can beneficially rebalance the biofilm ecology and reduce the severity of the bacterial attack on teeth could enhance the anti-caries physiochemical effects of fluoride.

Natural products, whether they be the milk-derived Casein Phosphopeptide-Amorphous Calcium Phosphate (CPP-ACP) or plant-derived phytochemicals, have the potential to maintain the delicate balance between inhibiting cariogenic virulence factors of plaque microflora without killing

'healthy' bacteria present in the oral microbiome. Oral care products containing CPP-ACP and phytochemicals would be particularly useful for individuals assessed to be at high caries-risk.

1.3 Casein Phosphopeptide-Amorphous Calcium Phosphate (CPP-ACP): Cariostatic effects

The tryptic digestion of milk casein has been shown to produce multi-phosphorylated casein phosphopeptides (CPP) that substantially increases its solubility and ability to stabilise calcium and phosphate ions, while still making the ions available to prevent demineralisation or promote remineralisation [Reynolds, 1987]. The anti-cariogenicity of CPP is ten times greater than that of the intact protein on a weight basis without the associated problems of unpalatability or allergenicity [Reynolds EC and Walsh LJ, 2005]. This natural process of calcium stabilization, transport, and delivery was used to develop a novel protein-based remineralisation technology called CPP-ACP that is now used globally in a number of oral care products.

CPP-ACP has several modes of cariostatic action. Firstly, it inhibits demineralisation of the enamel surface by providing a supersaturation of calcium and phosphate in acidic conditions [Reynolds, 1998]. Secondly, it enhances remineralisation of white spot lesions (WSL) by the precipitation of hydroxyapatite or fluorapaptite [Reynolds, 2008]. The pattern of CPP-ACP remineralisation is considered ideal as it results in significantly more subsurface remineralisation of the WSL compared to salivary-or fluoride-mediated remineralisation, while also depositing mineral that is significantly more resistant to future acid challenges [Cochrane et al., 2008]. Thirdly, there are reports that CPP-ACP can also have beneficial ecological effects on dental plaque through their ability to interfere with the colonisation of cariogenic mutans streptococci (MS) bacterial species [Rose, 2000b; Rose, 2000a]. This interference with oral microflora may have the potential to lessen the cariogenic potential of dental plaque by reducing the proportion of acidogenic and aciduric bacteria.

There is now a large body of robust evidence from randomised controlled trials (RCTs) and systematic reviews that demonstrate the anti-caries effects of CPP-ACP through its effects on the de-/remineralisation caries equilibrium [Krithikadatta et al., 2013; Li et al., 2014; Morgan et al., 2008; Rao et al., 2009; Yengopal and Mickenautsch, 2009]. There are relatively fewer clinical trials that support the *in vitro* ecological effects of CPP-ACP. Most of these trials show reduction in MS levels after using oral care products containing CPP-ACP [Emamieh et al., 2015; Plonka et al., 2013; Yetkiner et al., 2014]. A limitation of all the clinical trials investigating the microbial ecological effects of CPP-ACP is that they used salivary levels of single bacterial species (*S. mutans*) as the surrogate marker, while what is more relevant from the caries point of view is to know the numbers of a range of both cariogenic and healthy bacteria present in the dental plaque.

A drawback of the current commercially available CPP-ACP oral care product (GC Tooth Mousse) is that it is designed as a topical crème, meant to be applied on teeth after brushing with a regular fluoride dentifrice, and for best results the CPP-ACP crème should be allowed to remain in contact with teeth for as long as possible. This two-step process is likely to affect compliance particularly in young children. A new CPP-ACP oral care product was released commercially in mid-2017 called MI Paste® ONE. This is a toothpaste that combines CPP-ACP into a regular fluoride toothpaste and is designed to be used for routine twice-daily toothbrushing. The effects of MI Paste® ONE on caries risk is currently being tracked in a large-scale RCT (Universal Trial Number U1111-1173-5442). The next conceptual advance is to add cariostatic phytochemicals to this CPP-ACP toothpaste to improve its ecological effects on dental plaque, which is the rationale for this proposed study. Combining remineralising effects of CPP-ACP with the antimicrobial effects of natural polyphenols in a single oral product has the potential to become a new standard for oral care, especially for individuals assessed to be at high-risk of developing dental caries.

1.4 Natural phytochemicals in dental caries prevention

The term 'natural products' is generally used to refer to secondary metabolites or phytochemicals derived from plants, herbs, spices, and fruits. While not essential nutrients, these bioactive chemicals are believed to be responsible for much of the disease protection conferred by diets rich in plant-based products [Arts and Hollman, 2005]. The advent of the 'Antibiotic Era' in the mid-20th century shifted the emphasis away from natural product-based antimicrobial agents. However, the emergence of antibiotic-resistant bacterial strains and the adverse side-effects of many synthetic antibiotics has renewed interest in the search for more biocompatible natural antimicrobial agents, including those with actions against cariogenic biofilms.

Antimicrobial agents have been recommended for high caries-risk individuals based on the rationale that while fluoride can reduce the critical pH at which dissolution starts, effective antimicrobial agents can decrease the depth of the Stephan's curve pH drop following consumption of fermentable carbohydrates [Øgaard B, 2000]. An entire range of biocides and antibiotic agents have been used in the past to reduce MS levels and inhibit dental caries. However, most of these chemotherapeutic agents have a broad-spectrum of antimicrobial action causing an undesirable suppression of even the healthy plaque microflora. Furthermore, dentifrices and mouthwashes containing commonly used microbiocides like chlorhexidine or triclosan have also been associated with a number of adverse side-effects that may discourage their long-term use [Eley, 1999; Fiss et al., 2007]. Natural phytochemicals that display antimicrobial effects could be an appealing substitute to

traditional chemotherapeutics, as their better biocompatibility and low toxicity will make it easier for the general public to accept them.

Some natural phytochemicals have displayed minimum inhibitory concentrations almost comparable to chlorhexidine [Chung et al., 2006; Hwang et al., 2000]. However, the bacterial growth inhibitory effects of phytochemicals are generally not expected to be selective for specific cariogenic bacterial species [Jeon et al., 2011]. More promising are phytochemicals that despite lacking significant biocidal activity, can disrupt key cariogenic virulence properties like glucan synthesis or bacterial adhesion. Glucans play a critical role in early stages of cariogenic biofilm development by providing specific binding sites for bacterial adhesion and colonisation, along with providing the biofilm bulk, stability, and a reserve carbohydrate source for enhanced acid production [Bowen and Koo, 2011]. Phytochemicals that inhibit glucan synthesis or bacterial adhesion can reduce the total mass biofilm mass without affecting viability of oral bacteria. Animal model caries studies investigating the effectiveness of anti-biofilm phytochemicals have shown that fluoride-phytochemical combinations were as potent as fluoride-chlorhexidine controls in caries inhibition, with the added benefit that their antimicrobial effects did not disrupt the resident oral microbiota [Koo et al., 2010; Koo et al., 2005]. Evidently, natural products that can specifically target bacterial adherence, glucan synthesis, or biofilm matrix acidification are an attractive and appealing alternative to broad-spectrum microbicides [Jeon et al., 2009].

The laboratory phase of this research project evaluated the effects of natural products on cariogenic biofilms using *in vitro* biofilm models. Among the range of different plant extracts and phytochemicals tested, highly-purified organic cranberry extracts (Diana Foods, Champlain, Canada) containing 20% proanthocyanidins (PAC) showed the most significant effects on biofilm metabolism (assessed by XTT Reduction Assays), biofilm biomass (Crystal Violet Assay), biofilm structural organisation (assessed by Confocal Laser Scanning Microscopy), and microbial viability (assessed by CFU counts). Despite the lack of significant bactericidal activity, PAC treatment can potentially diminish acidogenicity within *S. mutans* biofilms most likely by disrupting overall bacterial metabolism. This confirmed previous studies that have shown the PAC flavonoids present in cranberry could inhibit MS glucan synthesis through its potent anti-glucosyltransferase activity [Duarte et al., 2006; Gregoire et al., 2007; Koo et al., 2010; Steinberg et al., 2004].

A limitation of studies investigating natural products for caries prevention is that most of the cariostatic effects of natural products have been demonstrated in *in vitro* studies. Recent reviews have confirmed that despite the high number of studies on the biological effects of natural products most of them were laboratory based, with just over a tenth of the studies in Phase IV clinical trials, and even

fewer have led to commercial products [Freires et al., 2015; Freires and Rosalen, 2016]. Hence, the clinical trial phase of this research project proposes to investigate whether toothpastes containing a combination of biocompatible natural cranberry flavonoids and CPP-ACP can beneficially modify the microbial ecology of oral plaque biofilms and thereby reduce the cariogenic virulence of dental plaque deposits on teeth.

2. HYPOTHESIS

It is hypothesised that the antimicrobial effects of cranberry extracts can significantly enhance the anti-caries effects of CPP-ACP through microbial ecological changes in dental plaque biofilms.

3. AIM OF THE STUDY

Determine any microbiological changes in the dental plaque of high caries-risk individuals using either MI Paste® ONE CPP-ACP toothpaste or the same toothpaste enriched with a cranberry additive.

4. OUTCOME MEASURES

The outcome measure to test the hypothesis is tracking the bacterial load numbers of 14 key bacterial species (this includes both cariogenic bacteria and health-associated bacteria).

5. PROJECT DESIGN

5.1 Approvals and consent

Prior to the commencement of this study, ethical approval will be obtained from the Human Research Ethics Committees (HREC) of The University of Queensland and Metro North Health Oral Health services for a Phase0/Pilot trial. The trial will be registered with the Australian Clinical Trials Registry. The CPP-ACP toothpastes proposed to be used in the clinical trial will be tested under the Clinical Trial Notification (CTN) scheme of the Australian Therapeutic Goods Administration (TGA). Site-specific assessment (SSA) approvals will be obtained for the Metro North Orthodontic Clinics located at the UQ Oral Health Centre, Herston. Biosafety approvals for processing the plaque samples in the UQ Oral Health Centre's Research Laboratory will be sought from The University of Queensland.

Written informed consent will be obtained from the participants or parents/legal guardians (if participants <18 years) who volunteer to take part in the trial. The aims of the research, what the potential participants are expected to do during the trial period, and the risks/benefits involved will be communicated both verbally and in a written form. The participants and parents will be allowed sufficient time to read the project description in non-scientific language, understand the recruitment strategy, and sign the consent forms if willing. Any doubts will be cleared before obtaining consent and

it shall also be informed that their decision to participate or not would not affect their continued dental care. The participants will also be given the option of dropping out of the trial at any time before completion if they express a wish to no longer participate.

5.2 Research participants and inclusion/exclusion criteria

Potential study participants will be initially identified based on whether they are undergoing fixed orthodontic treatment, as these patients are considered to belong to the high caries-risk group. The prospective participants will be approached by their supervising orthodontist to ascertain their initial willingness to be part of the trial. The following selection criteria will be used:

5.2.1 Inclusion criteria:

(i) A minimum of 10 years of age and at least 4 fully erupted permanent maxillary anterior teeth, (ii) Good general physical health as determined by a review of the medical history, (iii) Undergoing fixed orthodontic treatment in both arches, with treatment having been underway for at least 1 month, (iv) Available to attend a review appointment in 4 weeks, and (v) Not currently using antibiotics or any antibacterial/anti-plaque mouthrinses.

5.2.2 Exclusion criteria

(i) Any medical condition or disability preventing normal manual tooth brushing, (ii) Allergy to milk casein proteins or intolerance to any of the components of the CPP-ACP toothpastes, (iii) Unwillingness to use a fluoridated dentifrice, (iv) Untreated periodontal disease, (v) Clinical evidence of active caries.

5.3 Randomisation and sample size

At the completion of the recruitment appointment, participants will be assigned to one of three groups (two intervention groups and one active control group) by computer generated randomised allocation. For answering the hypothesis of this pilot trial, a statistical power analysis calculated that the total cohort of projected participants should be 60 (N=20 for each group), based on $\alpha = 0.05$ and a power of 95%. Recruitment of participants will aim for 30 per group to account for lack of compliance or any dropouts during the trial period.

The three trial groups are:

- Group A: participants in this group will receive the MI Paste® ONE CPP-ACP toothpaste.
- Group B: participants in this group will receive the 'All-in-one' CPP-ACP – Cranberry toothpaste.
- Group C: participants in this group will receive a standard fluoride toothpaste as the active control.

All the toothpastes will contain fluoride at concentrations that are consistent with standard care. Study participants in all three groups will be given standardised oral hygiene instructions and encouraged to

use the supplied toothpaste for routine twice-daily manual toothbrushing throughout the four weeks of the trial. Tubes of the toothpaste will be pre-weighed so that extent of compliance in using them can be assessed.

5.4 Blinding

The clinical trial is proposed to be double-blinded in nature. The intervention products (MI Paste® ONE and 'All-in-one' toothpastes) and the control toothpaste will be identical in appearance and flavouring. Packaging of the toothpastes will be marked with a digital code, but the content of the individual tubes will not be known to the participants, the clinicians, or the investigator. The codes will be provided to the chief supervisor of the study. Only after the statistical evaluation of all parameters is completed will the decoding occur.

5.5 Dental plaque sample collection and storage

Dental plaque samples will be collected at two time points - at a baseline visit just before prospective participants start using the trial toothpastes, and then at a follow-up visit that is scheduled after four weeks of using the supplied toothpastes. Before both the plaque collection appointments, the participants will be instructed to refrain from morning brushing and to avoid food/drink two hours prior to collecting the dental plaque swab sample.

The plaque sample will be collected before removing modules, chain or ligature-ties by swabbing the labial gingival third of the upper and lower teeth using a microbrush. The tips of the microbrushes will be cut off and immediately placed in 0.1ml of sterile 0.15M phosphate buffered saline (PBS) containing 0.01% thiomersal (Sigma-Aldrich, St. Louis, USA). The plaque samples collected in the UQ Oral Health Centre Orthodontic Clinics will be labelled with a re-identifiable case-number and temporarily kept in a fridge. For final storage, the samples will be transferred to a -80 ^{0}C freezer in the UQ Oral Health Centre Research Laboratory until their microbial analysis.

5.6 Microbial analysis

Molecular microbiological analysis of the collected dental plaque samples will be done using real-time quantitative polymerase chain reaction (qPCR) analysis, by 16S rRNA sequencing and subsequent microbial population characterisation. The experimental steps are detailed below:

5.6.1 ***Preparation and extraction of DNA from supragingival plaque samples***

Deoxyribonucleic acid (DNA) will be extracted from the plaque samples using the MO BIO Power Soil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, USA). The samples will be vortexed and the 0.1 mL sample and microbrush tip, will be put directly in bead tubes with 60µL of Solution C1. Samples will be

incubated at -65 ^{0}C for 10 min and then shaken horizontally at maximum speed for 10 min using the MO BIO vortex adaptor. The remaining steps will be performed as directed by the manufacturer. The quality and quantity of DNA will be determined using an Infinite® M200 Pro microplate spectrophotometer with NanoQuant Plate (Tecan, Männedorf, Switzerland). Extracted DNA will be stored at -20 ^{0}C until analysis.

5.6.2 *16S rRNA sequencing and bacterial load determination*

The bacterial load of 14 bacterial species or species groups (*Actinomyces odontolyticus*, *Actinomyces viscosus*, *Bifidobaceterium dentium*, *Capnocytophaga gingivalis*, *Lactobacillus acidophilus*, *Lactobacillus casei*, *Neisseria flava/subflava group*, *Scardovia Wiggsiae*, *Steptococcus salivarius*, *Streptococcus mitis/oralis/pneumoniae* group, *Streptococcus mutans*, *Streptococcus sanguinis*, *Streptococcus sobrinus*, *Veillonella dispar/parvula* group) will be determined using a custom-made qPCR array (16 x 24 format) (Qiagen, Hilden, Germany). Each assay targets the 16S rRNA gene of the relevant bacterium and are designed using the GreenGene database for 16S sequences.

The DNA sample will be mixed with a proprietary master mix (containing reaction buffer, nucleotides, reference dye, and HotStart® DNA polymerase) and dispensed into a 384-well plate (10 µL/well, 10 ng DNA/well) containing dried down primers and fluorogenic probes for each of the bacterial 16s rRNA genes tested. Reactions will be performed with the ABI-7900HT sequence detection system with the following cycling conditions: enzymatic activation at 95 ^{0}C for 10 minutes and then 40 cycles of 95 ^{0}C for 15 seconds and 60 ^{0}C for 2 minutes. Data will be analysed using the sequence detection system software (v2.4; Applied Biosystems, Carlsbad, CA, USA) and the Qiagen analysis centre portal (https://www.qiagen.com/au/products/genes%20and%20pathways/data-analysis-center-overview-page/). Arrays will also contain four synthetic template standards of known gene copy number, a pan-Bacteria assay that will detect total bacteria, a positive PCR control to test for the presence of inhibitors in the sample, and a non-template control to account for assay background. Cycle threshold (CT) values for each sample will be normalised to Pan Bacteria (bacterial load) and the gene copy number in each sample will be determined by comparing the CT values of each sample to those of the standards.

5.7 Statistical analysis

Exploratory data analysis will be performed to determine the most appropriate statistical test. The assumptions of equality of variances and normal distribution of errors will be checked. Comparisons will be made using analysis of variance (ANOVA) and levels of significance is set at 5%. For statistical analyses IBM SPSS Statistics version 24 (IBM, NY, USA) will be used.

5.8 Resource requirements

Cost of consumables required for plaque sample collection and laboratory microbial plaque analysis is approximately A$12000. The trial toothpastes will be supplied free of charge by GC Australasia for purposes of this research project.

The research project's expenses will be funded by the chief Investigator Prof. Laurence Walsh research grants and the UQ School of Dentistry Student Research Fund.

5.9 Timeline

Task	Expected task initiation	Expected task completion
Ethical approval from HREC of QH and UQ	Jan 2018	March 2018
SSA approval from Metro North	March 2018	May 2018
Participant Recruitment	June 2018	Aug 2018
Baseline plaque collection	July 2018	Sep 2018
End-point plaque collection	Aug 2018	Oct 2018
Microbial analysis of plaque samples	July 2018	Nov 2018
Data analysis/publications	Nov 2018	Dec 2018

7. Dissemination of findings

It is proposed that the findings of this research project will be submitted for publication in peer-reviewed dental journals and will also form part of a PhD thesis submitted to The University of Queensland. There are no restrictions on publishing the results of the study. None of the investigators involved in the study have any conflict of interests or commercial stake in any of the products being tested in the study.

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