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Biofilm inhibition and antimicrobial activity of a dentifrice containing salivary substitutes

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Dates:
Accepted 15 March 2007

To cite this article:
Int J Dent Hygiene **5**, 2007; 218–224
Hatti S, Ravindra S, Satpathy A, Kulkarni RD, Parande MV. Biofilm inhibition and antimicrobial activity of a dentifrice containing salivary substitutes.

Abstract: *Background/aims:* A healthy mouth harbours the continuous combined action of a salivary defence system with that of a salivary peroxidase system, containing lactoferrin, lysozyme, immunoglobulin and growth factors. This system maintains neutral pH and creates an oral environment where harmful bacteria are inhibited, thus preventing the formation of biofilms. The objective of this clinico-microbiological trial was to evaluate the anti-plaque effect of a dentifrice containing salivary substitutes, compared with a placebo-control dentifrice and to assess the effect of dentifrice on oral bacterial count. *Methods:* The design was a randomized controlled, double-blind, parallel study comparing a placebo-dentifrice to a dentifrice formulation containing salivary substitutes. Toothpaste slurry rinses were used over a 96-h period by 20 volunteers who refrained from all other oral hygiene procedures. Commercially available fluoride toothpaste was used as control. Plaque was scored and unstimulated salivary samples were collected at day 0 and after 4 days. A microbiological analysis was carried out for the salivary samples. Data were analyzed by using Student's *t*-tests. *Results:* There was a statistically significant mean difference in plaque scores after using test paste (1.19 + 0.31) in comparison with those using placebo toothpaste (1.95 + 0.33). The difference between mean increase in colony forming units for the test and the placebo group was $(25.2 + 8) \times 10^5$ and $(17.5 + 6.01) \times 10^5$, respectively, which was statistically significant. *Conclusions:* The findings of the study support the hypothesis that toothpaste containing salivary substitutes prevents dental biofilm formation and

exhibits antimicrobial property when compared with a placebo dentifrice.

Key words: biofilm inhibition; clinical trial; oral bacteria; plaque; salivary peroxidase system; salivary substitutes; toothpaste

Introduction

In spite of the changing concepts on aetiology and natural history of periodontal diseases over the last few decades, plaque control by self-care is still regarded as essential in the treatment and prevention of periodontitis (1). As most individuals seem to have difficulty in achieving perfect control by mechanical means, research has been directed towards the development of safe and effective chemical anti-plaque agents (2).

Many marketed products are multifunctional by virtue of a range of agents formulated into toothpastes. Use of therapeutic agents in toothpastes to produce an inhibitory action on plaque formation is now a well-established approach to improve gingival health (2–5). One of the earliest groups of compounds studied was metal salts (6, 7). Of these, salts of zinc and tin have received the greatest attention, more so because of their recognized antibacterial activity and their relative high safety profile (8–13).

While the quest for the most suitable antiplaque and antibacterial agent to be incorporated in a dentifrice formulation goes on, one such compound is a product which brings back the oral system naturally (Bioxtra®). It is a product of molecular technology which mimics human saliva and encourages better oral hygiene.

Ambient temperature and regular presence of moisture and nutrients make the mouth a paradise in which bacteria thrive and multiply. More than 70% of bacteria in the mouth are found in biofilms (plaque), yet oral biofilm bacteria have rarely been taken into consideration when developing oral hygiene products. Studies have shown that, due to the organized structure of biofilm, local concentration of antibiotics or chemical agents necessary to eliminate biofilm bacteria would need to be 1000 times higher than that for isolated bacteria (14–17). In a healthy mouth, the continuous combined action of a salivary defence system, such as salivary peroxidase system, lactoferrin, lysozyme, immunoglobulin and growth factors combined with good oral hygiene helps maintain neutral pH and creates an oral environment where harmful bacteria are inhibited, preventing the formation of biofilm.

The present study used a toothpaste which contained natural ingredients that form part of the salivary peroxidase system which maintains the mouth's natural ecosystem. The antimicrobial function of the test paste is the same as that present in saliva, tears, breast milk and other human and animal secretory fluids.

It was hypothesized that supplementing the natural ingredients of saliva through a dentifrice might prevent the formation of biofilm and help in preventing the progress of disease. The objective of this clinico-microbiological trial was two-fold: to evaluate the anti-plaque effect of a dentifrice containing salivary substitutes compared with a placebo-control dentifrice and to assess the effect of dentifrice on oral bacterial count.

Materials and methods

Experimental design and study population

A randomized controlled, double-blind, parallel study comparing a placebo-dentifrice to a dentifrice formulation containing salivary substitutes was conducted. All participants provided written informed consent. The inclusion criteria required patients to be at least 18 years of age, in good general health, dentate, with no visible signs of untreated caries and periodontal disease, without removable or fixed dental prosthesis or fixed and removable orthodontic appliances and with a high standard of oral hygiene and gingival health. A detailed medical history was obtained from subjects to exclude subjects on pharmacotherapy or with medical conditions, which might influence the conduct of the study. Twenty subjects of either gender volunteered to participate in the study. All received oral and written information concerning the study and satisfied the inclusion criteria. The study was conducted in accordance with the ethical principles that have their origin in the Declaration of Helsinki and that are consistent with Good Clinical Practice (Fig. 1).

Materials

The test toothpaste used for the purpose of the study was commercially marketed in the name of Bioxtra®. It is one of

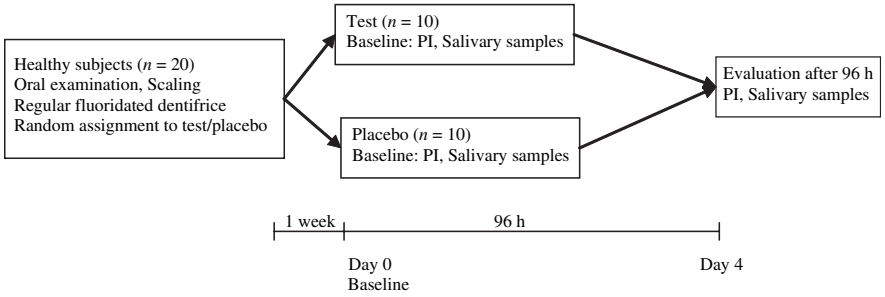

Fig. 1. Study design.

the natural ingredient-based products manufactured and marketed by Bio-X Healthcare, S.A. Company, Namur, Belgium. In addition to the common toothpaste ingredients like abrasives, humectants, sweetening and flavouring agents, Bioxtra® contains lactoferrin, lysozyme and lactoperoxidase. The placebo toothpaste was a commercially available fluoridated toothpaste marketed by Colgate-Palmolive, Mumbai, India. The study was self-funded by the authors and their institution.

Procedure

All subjects underwent professional scaling and polishing for all visible plaques following the use of a plaque disclosing agent and were asked to use a regular fluoridated dentifrice for 1 week before the start of the study. Plaque scores were recorded for all at day 1 (baseline score). Subjects were randomly allocated with (20 gm/80 ml) toothpaste slurry prepared by an independent pharmacist who was blinded to the study. Samples were to be used for 4 days. Samples were supplied in marked and coded amber coloured bottles to mask the visible hue. Subjects were to abstain from all forms of oral hygiene practices for 4 days and asked to rinse with randomly allocated slurries of dentifrices (placebo and test paste) twice daily. On day 5, all surfaces were scored for plaque index. Plaque was assessed using the Turesky *et al.* (33) modified plaque index. An average plaque score was calculated for each patient by summing the individual plaque scores for each tooth and dividing by the total number of sites scored for each subject. All clinical recordings were done by a single examiner who was blinded to the tooth paste sample.

Salivary sampling and microbiological procedures

Unstimulated salivary samples were collected from the subjects into wide-mouthed sterile bottles at day 1 (baseline samples) and day 5. The samples were coded and immediately sent for a microbiological analysis to the Department of Microbiology, SDM College of Medical Sciences & Hospital, Dharwad. All procedures were carried out using strict aseptic technique in a laminar airflow chamber (Kartos Int, Noida, New Delhi, India). Salivary samples were diluted in sterile normal saline to a final dilution of 1:100 000, 100 μl of this dilution was inoculated in Brain Heart Infusion Medium (BHI – calf brain, infusion from 200 g l^{-1}, beef heart, infusion from 250 g l^{-1}, protease peptone 10 g l^{-1}, dextrose 2 g l^{-1}, NaCl 5 g l^{-1}, disodium phosphate 2.50 g l^{-1}, agar 15 g l^{-1}, final ph 7.4 ± 0.2 at 25°C agar plates). The plates were inoculated at 37°C for 24 h. The colonies were counted using a digital colony counter (Sii Serwell Instruments Inc, Bangalore, India).

Statistical analysis

All the recorded data were separated according to the numerical code and allocated accordingly to the two groups. Evaluation was done with the Statistical Package of Social Science (SPSS) version 11. The significances of the clinical results within the groups and between the groups were tested with the Student's paired and unpaired *t*-tests. A value of $P \leq 0.05$ was considered statistically significant for all analyses.

Results

All volunteers completed the study. No adverse effects or any minor side effects occurred. The mean baseline plaque scores for placebo group was 1.36 ± 0.39 when compared with 1.59 ± 0.47 in the test group (Table 1). There was no statisti-

Table 1. **Mean plaque score comparing test toothpaste with placebo control toothpaste – plaque index**

Variable	Placebo toothpaste (n = 10), mean (SD)	Test toothpaste (n = 10), mean (SD)	95% CFI		*P*-value*
			Lower	Upper	
Baseline	1.36 (0.39)	1.59 (0.47)	−0.6331	0.1731	0.2462
96 h	3.31 (0.44)	2.78 (0.51)	0.0802	0.9798	0.0235
Difference	1.95 (0.34)	1.19 (0.30)	0.4568	1.0632	0.0001

*Student's paired *t*-test. CFI, confidence interval.

Table 2. **Mean salivary bacterial colony forming units (CFU) comparing test toothpaste with placebo control toothpaste – plaque index**

Variable	Placebo toothpaste (n = 10), mean (SD)	Test toothpaste (n = 10), mean (SD)	95% CFI Lower	95% CFI Upper	P-value*
Baseline	23.5 (5.6)	25.8 (6.65)	−8.0753	3.4753	0.4137
96 h	48.7 (11.11)	43.3 (10.84)	−4.9451	15.7451	0.2873
Difference	25.2 (7.89)	17.5 (6.0)	1.1078	14.2922	0.0245

*Student's paired t-test, CFI, confidence interval.

cally significant difference between test and placebo groups in the baseline scores (P = 0.2462). The mean plaque score using placebo toothpaste after day 4 was 3.31 ± 0.24, whereas that for test paste was 2.78 ± 0.51. There was a statistically significant difference between the two groups (P = 0.0235). The mean difference in plaque scores from baseline to day 4 in placebo group was 1.95 ± 0.33, whereas that in test group was 1.19 ± 0.31, again the values were found to be statistically significant between both groups (P = 0.0001).

The second part of this experimental study included counting of salivary in colony forming units (CFU) of oral bacteria at baseline and after using toothpastes for 4 days. The mean baseline CFU for placebo group was found to be $(23.5 \pm 5.6) \times 10^5$ CFU when compared with $(25.8 \pm 6.65) \times 10^5$ CFU in the test group (Table 2). There was no statistically significant difference between the placebo and test group for the baseline values (P = 0.4137). After 4 days of toothpaste use, the mean CFU values for the placebo and test groups were $(48.7 \pm 11.18) \times 10^5$ and $(43.3 \pm 10.84) \times 10^5$ respectively. The mean increase in CFU values for the placebo and test group was $(25.2 \pm 8) \times 10^5$ and $(17.5 \pm 6.01) \times 10^5$ respectively, which was statistically significant (P = 0.0245).

Discussion

The aim of our study was primarily to determine whether a toothpaste formulation containing salivary substitutes could provide a benefit at inhibiting plaque formation in comparison with benchmark control fluoride toothpaste (18, 19).

The study was not designed to demonstrate the efficacy of any particular active ingredient, but to show that the product as a whole was significantly better than a recognized conventional fluoride toothpaste. This is the first study to the best of our knowledge investigating the antiplaque efficacy of a dentifrice containing human salivary substitutes in individuals having normal salivary flow.

Mechanical plaque control is an established gold standard of all oral hygiene practices and has been clearly shown to retard the progression of gingivitis and periodontal disease (20). It has been shown that non-compliant patients exhibited signs of recurrent disease processes (21). The inconsistency of simple mechanical control of plaque accumulation has lead to a number of chemotherapeutic agents in dentifrices and mouth rinses. Independent studies have been carried out to determine the efficacy of active ingredients in toothpaste (18, 19, 22–25). These agents have generally been incorporated and the action of these agents has been focused on their anti-microbial action (26).

A 4-day plaque re-growth model instituted for this experiment has been previously used in numerous investigations and can be described as an established method for testing the plaque inhibiting effect of an oral hygiene product (18). The plaque re-growth model ensures a minimum standard of performance for any dentifrice with respect to plaque inhibition (27).

As the aim of our study was to evaluate the true chemical antiplaque effect of a dentifrice, the present study utilized only the use of toothpaste slurry and not the toothbrush. This is perhaps not surprising as studies attempting to assess the antiplaque effect of dentifrices are often hampered by the mechanical action of the toothbrush. The use of toothbrush would definitely influence the outcome of the study. This idea is consistent with that of Moran *et al.* (28) who pointed out that any antimicrobial product would prove effective when toothpaste is often used with a toothbrush.

In the present study, slurry was prepared by mixing the toothpaste in distilled water so that simple rinsing reproduces the quantity of active substances present in the oral cavity during normal tooth brushing without the mechanical cleaning effect. Previous studies followed 3 g 10 ml^{-1} regimen for making toothpaste slurries (18, 19). However, there is a practical difficulty to dispense small quantity of slurry to the subjects each time and allowing subjects to prepare slurry on their own would bring about increased variability. Therefore, 20 gm / 80 $ml,^{-1}$ slurry preparation was dispensed in our study to be used twice daily for 3 days. The amount of toothpaste used is greater than normally used on the brush, i.e. 1.45 g (29) but the resulting concentration in slurry (one in four) is similar to that achieved in the oral cavity (resulting from the dilution of the toothpaste on the brush by saliva).

Although the subjects recruited in the study were asked not to use any mechanical cleansing aid during the plaque re-growth model, the Hawthorne effect might still have influenced the outcome of the study. The Hawthorne effect refers

to changes in the behaviour of subjects that occur solely as a result of participation in an experiment. It is an important confounding variable in therapeutic trials, as a major behavioural factor that influences all outcome measures in periodontal studies is the degree to which subjects improve and maintain personal oral hygiene (30–32).

The indices were scored on all surfaces of the tooth. The use of Turesky *et al.* modified plaque index (33) to measure the effects on re-growth after 96 h cannot be extrapolated into effects on the diseases of gingivitis or caries, but may provide a rapid screening method for potential antiplaque agents. Plaque index and not plaque weight or plaque area was assessed in the present study. Plaque indices remain the principal assessment of clinical outcomes in clinical trials of toothbrushes and other methods of plaque removal. There appears to be no significant advantage in using plaque weight in periodontal clinical trials (34). The use of the Gingival Margin Plaque Index or plaque scoring by area to measure the effects on re-growth after 16 or 24 h cannot be extrapolated into effects on the diseases of gingivitis or caries, but may, as suggested (35), provide a rapid screening method for potential antiplaque agents.

An earlier *in vivo* study comparing the anti-biofilm and antibacterial activity of this toothpaste formulation reported significant reduction in quantity of plaque and salivary CFU (36). The study estimated the total number of viable bacteria by an ATP assessment of the bacteria versus the bacteria present. The positive results of Bioxtra® could be attributed to the lactoperoxidase, lactoferrin, and lysozyme, immunoglobulins and colostrum whey, which are present in the dentifrice as natural salivary substitutes. Van Hooijdonk *et al.* (37) reviewed the *in vivo* antimicrobial and antiviral activity of components in bovine milk and colostrums involved in non-specific defence, with special emphasis on lactoferrin and lactoperoxidase. VanSteenberghe *et al.* (38) demonstrated the protective effect of LP-s containing toothpaste in patients suffering from radiation-induced xerostomia. Patients treated with the test toothpaste showed less plaque formation and a lower incidence of gingival inflammation. Ingestion of food activates the bacteria in the mouth to produce hydrogen peroxide. Lactoperoxidase in Bioxtra® activates a reaction between hydrogen peroxide, thiocyanite and oxygen to produce hypothiocyanite which inhibits bacteria that disturb oral health thus maintaining a natural oral ecosystem. Lactoferrin is one of the most important naturally occurring antimicrobial agents in the mouth. It works in close conjunction with lysozyme to regulate conditions in the mouth. It is antibacterial (bacteriostatic as well as bactericidal), antiviral, and antiparasitic which is a key part of primary defence system and promotes commensal flora. The direct bacteriostatic effect is attributed to iron deprivation whereas bactericidal activity is related to the direct binding of lactoferrin to the microbial membrane, which alters the membrane permeability through dispersion of lipopolysaccharides and leads to microbial killing (37, 39, 40). Lysozyme is an enzyme present in saliva known to have antifungal effect and it has synergistic action with lactoferrin (41–43). Immunoglobulins are important proteins produced by the body's immune system. Colostrum whey contains a mixture of antimicrobial molecules with vitamins and calcium, which help safeguard health and vitality, protecting the mouth from disease.

Bioxtra® has been formulated keeping in mind the guidelines on the assessment of the efficacy of tooth pastes, which warrants a dentifrice to be non-allergic, having no bacterial resistance and safe for long-term use (44). Bacteria cannot become resistant as they do not become resistant to natural saliva. Also the flavouring agent is natural essential oils which have been carefully selected to be gentle on sore mouths. As a result there is less likelihood of allergic reaction. As it mimics the activity of natural saliva and contains no antibiotic or other strong chemicals, it may be suitable and recommended for regular long-term use. Shahdad *et al.* carried out a double-blind, crossover study comparing Bioxtra® systems with another in patients with post-radiotherapy xerostomia and reported a better acceptance and compliance in xerostomic patients with Bioxtra® systems (45).

In our study, there were statistically significant reductions in test group compared with control group. These differences in the overall changes in the indices and CFU suggest antibacterial effect in the test paste responsible for its antiplaque and antibacterial effect.

There is little evidence to support the association of groups of bacteria with plaque. To date, the studies describe complex and variable associations without being able to define the extent to which any species or combination of species can account for the clinical differentiation between health and disease (46–49). In this study we assessed only the quantitative (CFU) analysis and not the qualitative analysis, as plaque harbours a variety of micro-organisms and it is difficult to isolate, culture and identify each type of micro-organism separately. No subgingival or GCF samples were assessed as no effect on the subgingival microbiota will be expected from toothpaste (50). Also, the importance of the contained detergents must be considered as many surfactants, including those in commercial formulations, have been shown from *in vitro* studies to reduce plaque formation (51).

In addition, some of the subjects in our study showed lower than expected levels of plaque than seen in previous trials and therefore the use of different volunteers with differing tendencies for plaque re-growth does not make a direct comparison possible. It therefore remains to be determined whether the new formulations would be of value to gingival health irrespective of a success in this study to exhibit any effects on plaque formation and oral bacterial count. Therefore, further studies with larger sample size under the same or at least similar conditions are essential for conclusive evaluation, in particular in comparison with other active substances.

Thus, within the limitations of the study, it may be concluded that dentifrice containing human salivary substitute has a better antiplaque efficacy and antimicrobial property as compared with a placebo dentifrice.

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