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Development of methods to enhance extrinsic tooth discoloration for comparison of toothpastes

1. Studies in vitro

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Abstract

Background: The interaction of chlorhexidine with dietary chromogens to cause extrinsic dental staining has been exploited in vitro and in vivo to study tooth discoloration and its control. These studies in vitro investigated factors that might enhance stain formation, and evaluated formulations to inhibit the stain with the primary aim of devising a protocol for use in vivo.

Method: The standard method cycled acrylic specimens through saliva, 0.2% chlorhexidine and tea on the hour 8 times per day and stain was measured using a spectrophotometer. Test interventions were 3 ''whitening'' toothpastes (A, P, R), a fluoride toothpaste (C) and water. In studies 1 and 3 interventions were at 09:00 and 16:00, and in studies 2 and 4 at 09:00 and 13:00. Between cycles, specimens remained dry in studies 1 and 2 and were maintained in water day and night in studies 3 and 4. Studies 5–7 determined the influence of tea temperature, exposure time and concentration, and chlorhexidine temperature and exposure time on stain development. Studies 8–10 modified the standard procedure using tea at triple strength and 50°C, and assessed stain inhibition by toothpastes and water using optical density, colorimetric and visual assessment recordings.

Results: In studies 1–4, there were highly significant differences between interventions. Overall, the experimental whitening paste (P) produced the most stain inhibition, and water or the proprietary whitening paste (R), produced the least stain inhibition. More stain inhibition was seen with interventions at 09:00 and 16:00. Both tea concentration and temperature significantly influenced staining. Chlorhexidine temperature did not influence staining. Exposure time to tea and chlorhexidine had a small effect on staining. In studies 8 and 9, interventions at 09:00 and 16:00 were more effective; the most stain inhibition was with paste P and the least with water, paste R being intermediate. In study 10, P was the most effective and R the least effective interventions.

Conclusions: These studies in vitro suggest that the chlorhexidine tea stain model can be manipulated to enhance stain and thereby should improve discrimination between stain inhibition formulations. The timing of interventions in the model appears to be important. These studies in vitro were used to plan a clinical protocol.

Key words: chlorhexidine; extrinsic staining; tea; toothpaste; whitening

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Introduction

Relatively recently a considerable number of ''whitening'' toothpastes have appeared on the market. A cursory appraisal of the ingredients suggests that most are formulated to control extrinsic dental stain rather than to change the natural tooth colour through a bleaching action (Sharif et al. 2000). Some products also contain other agents to benefit dental health, notably for

caries, gingivitis and sensitivity. Despite the number of products, there is a dearth of classical randomised controlled clinical trials to support claims of efficacy against tooth staining. This is possibly not surprising since there is a limited understanding on the aetiology and, more particularly, the mechanism of tooth discoloration (for reviews, see Addy & Moran 1995, Watts & Addy 2001). Moreover, natural extrinsic tooth discoloration appears to vary markedly between individuals and is relatively slow to develop. Both of these make the design of clinical protocols difficult, which may reveal the comparative efficacy of ''whitening'' products. The task is made more complex with respect to toothpastes, which could influence staining through physical and chemical processes. The chemical effects could also be stain inhibitory, removal or both.

Perhaps, surprisingly, more is now understood concerning the aetiology and mechanism of tooth staining associated with the oral use of cationic antiseptics and certain metal salts compared with natural staining (for a review, see Addy & Moran 1995). The dietary theory of extrinsic tooth staining associated with such agents was propounded some years ago (Addy & Jenkins 1977) and later supported by a considerable number of laboratory and clinical studies (for a review, see Watts & Addy 2001). Arising from this research, it became apparent that the laboratory and clinical models could be manipulated to study a range of variables, including those influencing the rate of stain formation (Prayitno & Addy 1979), the activity of agents in formulations (Addy et al. 1989), the potential of new actives to cause staining (Addy & Roberts 1981a) and stain inhibition and removal by products and formulations (Sharif et al. 2000). As with many clinical protocols to evaluate treatments, the magnitude of the outcome variable developing under the placebo treatment can greatly influence the likelihood of showing a significant benefit of test formulations. Furthermore, the regimen of the use of agents may also be crucial to efficacy.

The forced chlorhexidine, tea-staining model has been used in a variety of clinical protocols to study factors influencing stain development and control (Addy & Roberts 1981a, Jenkins et al. 1989, Addy et al. 1991). The aims of the present studies in vitro were 3-fold and inter-related, namely to optimise extrinsic stain formation, to study the frequency and timings of interventions, and to plan a protocol in vitro that might predict similar outcomes in vivo.

Material and Methods

The methods all employed modifications of the original chlorhexidine tea staining on optically clear acrylic in vitro (Addy et al. 1979) and forced chlorhexidine tea staining *in vivo* protocols (Addy et al. 1991). The model in vitro cycles the acrylic substrate through saliva, 0.2% chlorhexidine and finally a standard tea solution, on the hour, 8 times per day (approximately 09:00–16:00 h). The acrylic specimens measured $3 \times 1 \times 0.5$ cm to fit the specimen chamber of an UV/visible spectrophotometer and used groups of 6 specimens for each treatment regimen. Baseline optical density readings were taken of all specimens against a standard specimen zeroed for the instrument. Optical density readings were performed at the end of each of the 8 daily cycles at the lambda maximum of 295 nm for tea. Besides optical density readings, some experiments recorded L, a, b values using a chromometer applied to the flat surface of the specimens and visual grading blind by a group of 6 individuals using the intensity scores of the Lobene (1968) stain index (0 = no stain, 1 = light stain, 2 = moderate stain, 3 = heavy stain). The standard operating procedure for each cycle during each experiment was as follows:

1. Groups of 6 acrylic specimens were placed in unstimulated saliva, from the same individual, for 2 min and removed into distilled water for 30 s.
2. Placed in 0.2% chlorhexidine solution for 60 s and removed to distilled water for 30 s.
3. Placed in a standard tea solution for 60 s.
4. Left dry until the next cycle.

At the end of the 8 cycles, the blocks were allowed to air dry and then read on the spectrophotometer. Daily cycles were performed until one particular treatment regimen achieved an optical density of $\geqslant 2.0$. Interventions within the staining procedure were toothpaste slurries prepared by thoroughly mixing 5 g of toothpaste in 20 ml of distilled water using a rotary mixer (water was used as the control intervention). The exposure of specimens to the slurries was for 120 s. Within the staining cycle, these interventions were used after saliva exposure but before chlorhexidine soaking. The standard tea solution was prepared by boiling 4 g of tea in 400 ml of distilled water for 4 min. The infusion was then decanted through gauze and allowed to cool to room temperature ($21 \pm 4^{\circ}$C). The variations to the standard procedure were:

1. Timings of the interventions.
2. Temperature of the standard tea and chlorhexidine solutions.
3. Concentration of the tea.
4. During and post cycle aqueous or dry environments.
5. Duration of chlorhexidine and tea exposures.

Unless otherwise stated, the interventions were:

1. Water (negative control).
2. Whitening toothpaste Product A.*
3. Whitening toothpaste Product R. †
4. Experimental whitening formulation P. ‡
5. Conventional fluoride toothpaste C.§

The following studies were planned:

Study 1 – dry

The standard operating procedure was used with the interventions immediately after saliva and before chlorhexidine at the 09:00 and 16:00 cycles. After each cycle, specimens were kept in a dry environment. Optical density was recorded after the 16:00 cycle. The study continued until a treatment group reached an optical density of $\geqslant 2$.

Study 2 – dry

Identical to study 1, but with interventions at the 09:00 and 13:00 cycles.

Study 3 – aqueous

As study 1, but specimens were maintained in water between cycles and overnight.

*Aquafresh Whitening, GlaxoSmithKline Consumer Healthcare, Weybridge, UK.

†Rembrandt Whitening, Den-Mat Corporation, Santa Maria, California, USA.

‡GlaxoSmithKline, Consumer Healthcare, Weybridge, UK.

§Colgate Regular, Colgate Palmolive, London, UK.

Study 4 – aqueous

As study 2, but specimens were maintained in water between cycles and overnight.

Study 5 – tea and chlorhexidine temperature

The study comprised 3 experiments all identical to the placebo control treatment of study 1 except that the first experiment used tea at 50°C, the second used chlorhexidine at 50°C, and the third both tea and chlorhexidine at 50°C.

Study 6 – tea concentration

The study was identical to the placebo control treatment of study 1, but the tea concentrations were 2 × standard, i.e. 2 g 100 ml^{-1} water, and 3 × standard, i.e. 3 g 100 ml^{-1} water.

Study 7 – duration of chlorhexidine and tea exposure

The study was identical to the placebo control treatment of study 1, but both the chlorhexidine and tea exposures were 2 and 4 min in duration.

Study 8 – clinical model in vitro

This study distilled the findings of studies 1–7 in order to compare 3 interventions, namely toothpaste R, toothpaste P and water. The modifications to the standard operating procedure of study 1 were as follows:

1. Triple-strength tea.
2. Tea at 50°C.
3. Interventions at 09:00 and 13:00 h.
4. Aqueous: spectrophotometric, chromometer and visual assessments were made of specimens.

Study 9

This study was identical to study 9 and compared the same interventions, but the timings of the interventions were at 09:00 and 16:00 h.

Study 10

This study was identical to study 9 in all aspects, except that all 5 interventions were tested, namely toothpastes A, R, P and C and water as the placebo control.

Statistical analysis

Logistically, it was impossible to run all studies simultaneously. Even though the standard operating procedure was standardised as much as possible, analyses were primarily within studies. Between- and across-study analyses were used when felt appropriate; otherwise descriptive statistics were used to draw conclusions across the studies.

Studies 1–4 together form a 2-way factorial structure, in each of which 30 specimens were allocated to the same 5 interventions. Baseline readings for each specimen were subtracted from each subsequent optical density reading. Within each study, optical density data were available for each day until one treatment reached a mean absorbance of 2.0; however, to avoid multiple comparisons the final day data were used in the intra-study analyses. Each study also ran for different numbers of days but included day 3 for all studies: these data were used for inter-study analyses. For each study, analyses of variance for differences between treatments were conducted at day 3 and the respective final measurement day. Significant unpaired *t*-tests were performed. To avoid multiple paired comparisons, these were restricted to those of the pre-study determined to be of prime interest, namely differences between the experimental paste P and the other treatments. Descriptive statistics were used to rank the order of effect from low to high stain. Analysis at day 3 to compare the interventions across the 4 studies fitted a 2-way analysis of variance with factors ''study'' ($n = 4$) and ''agent'' ($n = 5$) together with interactions between factors. To evaluate the specific paired study differences for times of intervention in non-aqueous (studies 1 and 2) and aqueous (studies 3 and 4) conditions, two-way analyses of variance were performed for data at days 3 and 5, respectively. Studies 5–7 were analysed using unpaired *t*-tests to compare the individual parameter of the particular experiments. In studies 8 and 9, optical density data were assessed for overall treatment effects using analysis of variance followed by unpaired *t*-tests. Visual assessment data were averaged over examiners, and differences between treatments were determined using Mann–Whitney tests. Study 10 was evaluated for treatment differences using Kruskall–Wallis tests followed by non-parametric Mann–Whitney paired tests.

Results

Studies 1–4

The means and standard deviations of the optical density on the final day of each experiment and at day 3 are given in Tables 1 and 2, respectively. The data are presented without adjustment for baseline optical density (average range 0.03–0.05), but analyses were based on data adjusted for baseline. The data were normally distributed and transformation of scale was considered to be inappropriate.

Table 1. Mean (standard deviation) optical density of specimens in studies 1–4 at respective exit days [ ] for the test toothpastes and water

Study [exit day]	1 [3]	2 [4]	3 [5]	4 [6]
water	2.01 (0.22)	1.76 (0.18)	0.88 (0.09)	1.08 (0.05)
TP.A	1.05 (0.15)	1.08 (0.18)	1.06 (0.09)	1.47 (0.09)
TP.R	1.77 (0.18)	2.26 (0.18)	2.04 (0.29)	2.40 (0.18)
TP.P	0.44 (0.18)	0.68 (0.12)	0.54 (0.17)	0.47 (0.10)
TP.C	1.28 (0.16)	1.47 (0.11)	0.83 (0.07)	1.31 (0.20)

TP = toothpaste.

Table 2. Mean (standard deviation) optical density of specimens in studies 1–4 at day 3 for test toothpastes and water

Study	1	2	3	4
water	2.01 (0.22)	1.77 (0.13)	0.51 (0.08)	0.50 (0.03)
TP.A	1.05 (0.15)	1.46 (0.27)	0.78 (0.10)	0.72 (0.03)
TP.R	1.77 (0.18)	1.97 (0.13)	1.28 (0.17)	0.82 (0.14)
TP.P	0.44 (0.18)	0.68 (0.06)	0.45 (0.17)	0.31 (0.07)
TP.C	1.28 (0.16)	1.26 (0.12)	0.50 (0.09)	0.55 (0.10)

TP = toothpaste.

In studies 1–4 least staining was noted with toothpaste P, and in all but study 1 most staining occurred with toothpaste R. Within-study analyses at day 3 for studies 1 and 2 and the respective end day for each study reveal overall highly significant treatment effects in every case ($p \ll 0.001$). Paired analyses of toothpaste P with all other treatments at day 3 for all studies and completion days for the respective studies were significant ($p < 0.01 - \ll 0.001$).

At day 3 for both study 3 and 4, within-study analyses revealed highly significant treatment effects ($p \ll 0.001$). Paired analyses in study 3 revealed toothpaste P to be significantly different from A and R ($p < 0.01$ and < 0.001, respectively) and in study 4 from all other treatments ($p \ll 0.001$). On completion, days 5 (study 3) and 6 (study 4) treatment effects were highly significant ($p \ll 0.001$). Paired analyses revealed toothpaste P to be significantly different from the all other treatments in both studies (p ranged from < 0.05 to $\ll 0.001$).

Based on day 3 data (Table 2), analysis across the studies revealed highly significant effects for study ($p \ll 0.001$), agent ($p \ll 0.001$) and interaction between the two factors ($p \ll 0.001$). Of the effects considered the least dominant appears the influence of the regimen timings of 09:00 and 16:00 (studies 1 and 3) and 09:00 and 13:00 (studies 2 and 4). Completion days for study pairs 1 and 2, and 3 and 4 are adjacent and therefore can be compared with days 3 and 5, respectively (Table 3). In most cases, in mean terms more staining was seen with interventions at 09:00 and 13:00 than at 09:00 and 16:00. Two-way analysis of variance and interactions support this observation, with timing effects highly significant for studies 1 and 2 ($p < 0.01$ and < 0.001 respectively) but not studies 3 and 4 ($p = 0.976$). Treatment and interaction effects were both highly significant for the three paired study comparisons ($p < 0.001$).

Studies 5–7

The appropriate optical density data for the above studies are shown in Table 4. Within the logistical constraints of the standard procedure, studies 5–7 were conducted at the same time and therefore analysed as one study. The data from study 1, day 1 have been added to Table 4. The standard operating procedure for study 1 was the same as studies 5–7; however, the time separating this study from the others was such that statistical comparisons were considered inappropriate. Only observational comparisons were therefore made. In studies 5–7, observational comparisons, including with study 1, reveal that increasing the temperature of tea alone or both tea and chlorhexidine to 50°C increases staining. Increasing chlorhexidine temperature to 50°C alone has little effect on staining. Tea concentration also considerably increases staining compared with the standard concentration. Thus, mean staining considerably increases from the standard concentration (study 1) through 2 × standard to 3 × standard. Exposure time to chlorhexidine and tea appears to have a small mean effect. Analyses for pairs of treatment regimens revealed significant differences for more staining with: tea alone or tea and chlorhexidine at 50°C over chlorhexidine alone at 50°C ($p < 0.05$); triple strength tea compared with double-strength tea ($p < 0.05$); and 4-min chlorhexidine tea compared with 2-min chlorhexidine and tea ($p < 0.05$). There was no significant difference between tea at 50°C compared to both chlorhexidine and tea at 50°C ($p > 0.05$).

Study 8

The mean (standard deviation) optical density readings, the colorimetric readings and the visual assessments for each of the interventions on day 1 are shown in Table 5. For optical density, the overall treatment effects were highly significant ($p \ll 0.001$) and paired comparisons showed highly significantly less stain with P compared with R and water: there were no differences between R and water. Colorimetric data ranked staining with P < W < R, but were not robust enough for meaningful statistical analysis. Visual assessments analysed by Mann–Whitney tests gave highly significant differences between products with a ranking of less stain with P < R < W.

Study 9

The mean (standard deviation) optical density, colorimetric and visual assessment results are shown in Table 6. Analysis of optical density data revealed highly significant treatment differences ($p \ll 0.001$), with P significantly less than R and W ($p < 0.001$). Colorimetric data were again not analysed for the same reason, but observationally the order was P < R < W. Treatment effects were again highly significantly different by visual assessment ($p < 0.001$) with the order P < W < R.

Study 10

The mean (standard deviation) optical density, colorimetric and visual assess-

Table 3. Mean (standard deviation) optical density of paired studies 1–4 on days 3 and 5 respectively, for the test toothpastes and water

	Day 3		Day 5	
	study 1	study 2	study 3	study 4
water	2.01 (0.22)	1.77 (0.13)	0.88 (0.09)	0.84 (0.47)
TP.A	1.05 (0.15)	1.46 (0.27)	1.06 (0.09)	1.13 (0.09)
TP.R	1.77 (0.18)	1.97 (0.13)	2.04 (0.29)	1.81 (0.17)
TP.P	0.44 (0.18)	0.68 (0.06)	0.54 (0.17)	0.36 (0.03)
TP.C	1.28 (0.16)	1.26 (0.12)	0.83 (0.07)	1.07 (0.18)

TP = toothpaste.

Table 4. Mean (standard deviation) optical density of specimens for studies 5–7 for effects of temperature, tea strength and exposure time after 1 day of 8 cycles

Treatments	Optical density
[5] tea 50°C	1.30 (0.12)
[5] CHX 50°C, tea 50°C	1.29 (0.14)
[6] tea × 2 strength	1.59 (0.20)
[6] tea × 3 strength	2.11 (0.31)
[7] CHX 2 min, tea 2 min	0.79 (0.87)
[7] CHX 4 min, tea 4 min	0.88 (0.75)
[5] CHX 50°C	0.63 (0.16)
comparitor data study 1	0.60 (0.17)

[ ] study number; CHS: chlorhexidine.

Table 5. Mean (standard deviation) optical density (OD), colorimetric (E^*ab) and visual assessment (VA) readings for specimens in study 8 for the test toothpastes and water

	O	E^*ab	VA
water	2.19 (0.14)	31.30 (7.70)	2.22
TP.R	2.03 (0.10)	21.76 (3.56)	3.33
TP.P	1.06 (0.15)	16.37 (5.77)	2.00

TP = toothpaste.

Table 6. Mean (standard deviation) optical density (OD), colorimetric (E^*ab) and visual assessment (VA) readings for specimens in study 9 for the test toothpastes and water

	OD	E^*ab	VA
water	2.70 (0.20)	10.07 (2.71)	2.83
TP.R	1.66 (0.10)	7.14 (1.45)	3.31
TP.P	0.37 (0.17)	6.68 (3.96)	1.08

TP = toothpaste.

Table 7. Mean (standard deviation) optical density (OD), colorimetric (E^*ab) and visual assessment (VA) readings for specimens in study 10 for the test toothpastes and water

	OD	E^*ab	VA
water	2.09 (0.13)	30.90 (5.76)	2.83 (0.11)
TP.A	0.45 (0.07)	3.38 (2.93)	1.03 (0.07)
TP.R	1.91 (0.17)	31.66 (4.54)	2.03 (0.07)
TP.P	0.42 (0.05)	3.87 (2.31)	0.86 (0.13)
TP.C	1.52 (0.14)	10.91 (3.70)	1.78 (0.39)

TP = toothpaste.

ment readings are shown in Table 7. In mean terms there is almost perfect agreement for the order of staining from least to most of P < A < C < R < W, with W and R and A and P reversed by colorimetry but where the actual differences between the pairs are very small and statistically not significant. In this study, to avoid making assumptions on distribution of data and concerns over heterogeneity, non-parametric analyses were performed. For each method, the Kruskal–Wallis test revealed highly significant treatment differences ($p \ll 0.001$). Paired analyses showed significant differences between all treatments by the three measurement methods, except A and P by optical density and colorimetry, W and R by colorimetry, and R and C by visual assessment ($p > 0.01$). The majority of the probability values were $p = 0.002$ (20 out of 26) and the remainder were p ranging from < 0.05 to < 0.01.

Discussion

Previous experience from clinical trials had suggested that failure to show extrinsic stain inhibition by an agent or formulation by comparison with a negative control was, at least in part, due to insufficient stain development. This report is made up of the findings from 10 studies in vitro, which were concerned with optimising a standard protocol in the expectation of clinical application. The standard protocol was manipulated in each study to assess the influence on staining of individual variables and in studies 1–4 and 8–10 to compare the chemical, stain inhibitory activity of a number of toothpastes and water. The chlorhexidine/tea-staining model was standardised as much as possible. A commercial chlorhexidine mouthrinse product was used; the same brand of tea and infusion method was consistent except when modified for specific studies and saliva from the same individual was employed throughout all experiments. The latter was considered necessary because of the reported significant effect of using different saliva sources in the model (Sheen et al. 2001).

One would expect little or no variation in the chlorhexidine rinse, but variation may come from both the tea preparation and the saliva. The pattern of results suggested however that if such variation existed, this was small by comparison with the influence of the variables tested in the respective studies. The number of studies made it logistically impossible to conduct more than a few studies together. Accepting that the method was standardised but that variation between experiments conducted at different times could have occurred, most analyses and conclusions are based on individual studies. When considered appropriate, across-study analyses were performed, but an observational appraisal of the data gives a very strong and consistent pattern for effects on which conclusions can be drawn.

In terms of the model, the variables tested had different magnitudes of effect on stain outcome. The timings of the interventions had a small but consistent, and in some experiments a statistically significant, influence, with the inhibition greater with interventions at 09:00 and 16:00 compared with 09:00 and 13:00, particularly for toothpaste P. This is, perhaps, not surprising since, within the model, 09:00 and 16:00 fit more closely with morning and evening toothbrushing. Also, it is likely that, through detergents and other ingredients in the toothpastes, stain removal as well as stain inhibition would occur. Analyses across studies 1–4 on day 3 also suggest a significant effect of dry versus aqueous environments, although this is as easily appraised by observation of Table 2. Thus, for most interventions, except for toothpaste P, there was always less stain in the aqueous environments compared with dry. This is perhaps not surprising, since conducting the studies in water to mimic to some degree the oral environment would result in some stain dissolution from the surface.

Increasing the concentration of the tea solution to two and three times the standard concentration significantly increased staining. Within the model this has not been tested previously, but would have been expected to occur for two possible reasons. Firstly, the rate of reaction between locally adsorbed chlorhexidine and the tea should increase with increasing tea concentration. Secondly, tea naturally causes staining by uptake onto and/or into the pellicle, albeit by an ill-understood mechanism (for a review, see Watts & Addy 2001). Again, increasing the concentration of the chromogen would be expected to increase surface deposition.

When observationally the data from studies 1 and 5 are compared, raising

the temperature of the tea had a considerable and significant effect. Raising the temperature of chlorhexidine alone had no effect on staining but when both tea and chlorhexidine were at 50°C again staining, increased, but only to a level similar to tea alone at 50°C. These data clearly indicate that it is the tea and not the chlorhexidine temperature that is dominant. This would be consistent with the known pattern of chlorhexidine adsorption to surfaces. At high concentration, an unstable multilayer is formed, which following exposure to an aqueous environment desorbs to leave a stable mono-layer (Emilson et al. 1973, Addy & Roberts 1981b). Temperature would be unlikely to change the adsorption pattern of the chlorhexidine. On the other hand, temperature would increase the rate of reaction of the tea with the adsorbed chlorhexidine, a feature noted previously for chlorhexidine and polyvalent metal salts such as tin and iron (Addy et al. 1985).

The duration of chlorhexidine tea exposures increased the staining, albeit proportionately to a small degree. Again, this might have been predicted since early studies on chlorhexidine adsorption *in vivo* indicated that uptake was rapid during the first 30 s and then slowed down, tending to plateau by 60 s (Bonesvoll et al. 1974). Exposing specimens for 2 and 4 min to chlorhexidine would therefore be unlikely to increase the amount of chlorhexidine on the surface to react with the tea. It is likely therefore that the slight increase in staining reflects a time-based effect of tea uptake by chlorhexidine and into or onto the pellicle.

The second aspect of studies 1–4 was to compare five treatments. Both within and between studies, there were highly significant treatment effects revealing greater stain inhibition by toothpaste P compared with all other treatments and reaching significance in almost every paired comparison. Conversely, toothpaste R resulted in more staining than other treatments or was second to water. The rather poor performance of toothpaste R is difficult to explain since ingredients common to the conventional paste C would be expected in paste R. Whatever, it would appear that actives specifically added to aid stain control in paste R were not displaying any significant activity in this model.

From the data derived from studies 1–7, modifications to the model were made, which were felt could be applied clinically. These modifications were designed to enhance staining, to increase the discrimination between treatments and to encourage the greater effect of the interventions on the stain. Therefore, studies 9 and 10 used triple-strength tea at 50°C with interventions at 09:00 and 16:00 and under aqueous conditions. Study 8 was similarly conducted, but with interventions at 09:00 and 13:00. The studies also used colorimetric measurements and visual assessments. Again, in all three studies and by all measurement methods, toothpaste P was statistically significantly and/or numerically superior for stain inhibition than all the other interventions. Toothpaste R appeared little different from water for effects on stain. Intervention time again appeared to be relevant, with greater mean effects with 09.00 and 16.00 timings compared to 09.00 and 13.00. The method and intervention used in study 9 were planned for application in an identical study *in vivo*.

In conclusion, staining can be enhanced to a considerable extent by increasing the temperature and concentration of the chromogen. The temperature of chlorhexidine and the duration of use of both tea and chlorhexidine have little or limited effects respectively, and could not be applied easily within the clinical model. The timings of interventions have a small but significant effect on the efficacy of treatments for stain inhibition.

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