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# Study Title: An *In Vitro* Study to Evaluate the Ability of a Toothpaste Formulation to Repair and Protect Demineralised Enamel Samples

## Analytical Objectives

- To use artificially demineralised human enamel samples as the test substrate to compare the ability of a toothpaste formulation, versus a negative control (Deionised water) to repair and protect the demineralised enamel samples during a 3 day treatment and pH cycling protocol.
- To use changes in the surface microhardness of the enamel samples to assess the ability of the treatments to repair and protect the demineralised human enamel samples.

## Study Overview

*In vitro* pH-cycling models are used to compare the ability of test articles to repair and protect tooth enamel, by mimicking test article treatments, saliva immersions and dietary erosive challenges. There are a variety of pH cycling protocols available within the literature, with this treatment protocol being based upon Huang *et al*. (2009)[1].

The study aimed to compare the ability of a toothpaste formulation versus a negative control (deionised water) to repair and protect human enamel samples during 3 days of treatment and pH cycling. The study has been summarised below.

Human enamel blocks were sectioned from acellular human teeth, lapped until their surfaces were flat and then highly polished. The preparation steps produced enamel specimens that were suitable for surface microhardness assessments.

The baseline surface microhardness of the enamel samples was measured using a calibrated Wilson Tukon 1202 microhardness indenter and Knoop diamond indenter tip. Enamel samples that met the baseline surface microhardness criteria progressed to the next stage of the study.

Enamel samples were demineralised in a demineralisation solution, for 1 hour at 37 °C, to produce early stage lesions in the enamel surfaces. The post-demineralisation surface microhardness of the enamel samples was measured using a calibrated Wilson Tukon 1202 microhardness indenter and Knoop diamond indenter tip.

Twenty enamel samples were stratified between two treatment groups, prior to treatment using the post-demineralisation surface microhardness values of the enamel samples (10 samples per treatment group).

The enamel samples in each treatment group were attached to a modified sample holder and subjected to 3 days of treatment and pH-cycling designed to mimic the oral environment. Following treatment, the surface microhardness of the enamel specimens was measured using a calibrated Wilson Tukon 1202 microhardness indenter and Knoop diamond indenter tip.

The percentage surface microhardness recovery (% SMHR) values achieved by the toothpaste formulation and the negative control were calculated and statistically compared. Higher %SMHR values were indicative of greater repair and protection of the demineralised enamel samples.

**Test Products**

**Table 1: Treatments**

| Treatment Group | Raw Chemical | Solution Prepared |
| --- | --- | --- |
| 1 | Carifree® Pro Gel 5000 - 1.1% Neutral Sodium Fluoride Anti-Cavity Toothpaste<br>Crimp Code: 682213<br>Exp: 04/24 | 1:1.6 Paste to DI water ratio (w/w)<br>Paste: 39.22g<br>DI Water: 62.75g |
| 2 | Negative Control - Deionised Water<br>Lot N°: 846747<br>Exp: 10/06/23 | Used as supplied by SLS. |

**Block Preparation**

20 enamel blocks, measuring 4 x 4 mm, were cut from acellular human teeth, and lapped planar-parallel using a Logitech PM5 lapping machine. The enamel surfaces of the samples were machine-polished using a MetPrep Saphir 550 to a final finish of 0.3 microns. One corner of each block was removed to ensure correct orientation of the samples on the SMH machine.

The enamel samples were sonicated, and surface checks were performed with an objective lens of the Wilson Tukon 1202 microhardness indenter. Suitable enamel samples were stored refrigerated, inside individual 7 ml Sterilins, on tissue dampened with 0.1 % thymol solution.

**Baseline SMH Measurements**

The baseline surface microhardness of the enamel samples was measured using a calibrated Wilson Tukon 1202 microhardness indenter and Knoop diamond indenter tip.

The enamel samples were orientated with the abraded corner in the top right. Baseline surface microhardness indents were placed in the centre of the enamel samples. Five Knoop indents were placed in a vertical line (under a load of 50 g, for 10 seconds). Surface imperfections were avoided.

Each enamel block was required to have an average baseline Knoop SMH > 250.0 HK (Hardness Knoop) and a standard deviation of < 20.0 to progress to the demineralisation phase of the study.

**Initial Enamel Block Demineralisation**

A demineralisation solution was prepared according to the following specifications:

- 50 mM Acetic acid
- 2.2 mM Calcium nitrate
- 2.2 mM Potassium phosphate monobasic
- 0.1 ppm Sodium fluoride
- Final pH of the solution was adjusted to 4.5 with sodium hydroxide.

Enamel samples were mounted onto acetate squares using double-sided tape. Enamel samples were immersed in 8 ml demineralisation solution per sample and placed into an oven for 1 hour (37 °C). The enamel samples were removed from the demineralisation solution, rinsed with deionised water for 2 minutes, and returned to their original containers.

**Post-demineralisation SMH Measurements**

The post-demineralisation surface microhardness of the enamel samples was measured using a calibrated Wilson Tukon 1202 microhardness indenter and Knoop diamond indenter tip.

The enamel samples were orientated with the abraded corner in the top right. Post-demineralisation Knoop surface microhardness indents were placed approximately 100 microns from the baseline indents. Five Knoop indents were placed in a vertical line at each timepoint (under a load of 50 g, for 10 seconds). Surface imperfections were avoided.

**Removal of Outliers and Stratification**

Specimens with either 'outlier' baseline/demineralised SMH values or standard deviations > 4.0 were removed from the study. Twenty blocks were stratified between 2 treatment groups ($n$ = 10) to create groups with similar mean post-demineralisation surface microhardness values.

**Treatment and pH Cycling**

The enamel samples in each treatment group were subjected to 3 days of the following treatment and pH cycling regime:

| Duration | Treatment & pH Cycling Regime |
| --- | --- |
| 5 min | Specified treatment |
| 55 min | Remineralisation solution |
| 5 min | Specified treatment |
| 55 min | Remineralisation solution |
| 60 min | Demineralisation solution |
| 2 hr | Remineralisation solution |
| 5 min | Specified treatment |
| 55 min | Remineralisation solution |
| 5 min | Specified treatment |
| Overnight | Remineralisation solution |

All pH cycling took place inside a hotbox heated to 37°C with the samples agitated during all treatment and immersions.

**Post-treatment (SMH) Measurements**

The post-treatment surface microhardness of the enamel samples was measured with a calibrated Wilson Tukon 1202 microhardness indenter and Knoop diamond indenter tip.

The enamel samples were orientated with the abraded corner in the top left. Post-treatment Knoop surface microhardness indents were placed approximately 100 microns from the baseline indents. Five Knoop indents were placed in a vertical line at each timepoint (under a load of 50 g, for 10 seconds). Surface imperfections were avoided.

**Data Management**

The day to day running of the study was documented in laboratory notebooks, which contained the study number 150-LAB-SMH-22-002. All laboratory notebook pages were signed by the study analysts.

The formula used to calculate the %SMHR values can be seen below:

%SMHR = (Post treatment SMH – Post Demin SMH) / (Baseline SMH – Post Demin SMH) * 100

All Excel datasets and formulas used in this study were entered, merged, and checked in accordance with data management SOPs. All data-check records were scanned into an electronic study folder.

A copy of the Excel dataset can be seen in Appendix 1.

**Statistical Analysis**

Minitab 18 was used to calculate descriptive statistics (mean, standard deviation, median, minimum, maximum, and sample size) for the %SMHR data achieved by each treatment.

A 2-Sample t-Test was selected to make pairwise statistical comparisons between the %SMHR values achieved by the two treatments. A copy of the statistical analysis report can be found in Appendix 2.

## Results

The mean percentage %SMHR values achieved by each treatment after 3 days of treatment and pH cycling is shown in Table 2.

**Table 2: Post Treatment Mean %SMHR**

| Treatment | Mean %SMHR | StDev |
| --- | --- | --- |
| Carifree® Pro Gel 5000 | 62.99 | 6.94 |
| DI Water | 37.64 | 12.90 |

After 3 days of treatment and pH cycling, the mean %SMHR value achieved by the Carifree Pro Gel toothpaste formulation was 63%, versus 38% for the negative control. The mean %SMHR value achieved by the Carifree Pro Gel toothpaste formulation was approximately 67% higher than the mean %SMHR value achieved by the negative control.

Statistical analysis of the %SMHR data showed that the Carifree Pro Gel toothpaste formulation achieved a statistically significantly higher %SMHR value than the negative control.

## Conclusions

The %SMHR data showed the Carifree Pro Gel toothpaste formulation was able to achieve statistically significantly greater repair and protection of the enamel samples than the negative control during this treatment and pH cycling study.

## Reference

1. Huang, S. B., Gao, S. S., & Yu, H. Y. (2009). Effect of nano-hydroxyapatite concentration on remineralization of initial enamel lesion *in vitro*. *Biomedical Materials, 4*(3), 1-6. doi:10.1088/1748-6041/4/3/034104

## Appendix 1: Surface Microhardness Raw Data

| Sample Number | Treatment Group | Baseline | | | | | | | Post Demin | | | | | | | | Post Treatment | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | HK1 | HK2 | HK3 | HK4 | HK5 | Average | StnDev | HK1 | HK2 | HK3 | HK4 | HK5 | Average | StnDev | Change in SMH | HK1 | HK2 | HK3 | HK4 | HK5 | Average | StnDev | Change in SMH | % SMHR |
| 002 | Carifree® Pro Gel 5000 | 299 | 326 | 299 | 332 | 323 | 315.80 | 15.67 | 68 | 63 | 71 | 69 | 67 | 67.60 | 2.97 | -248.20 | 239 | 207 | 227 | 225 | 224 | 224.40 | 11.44 | 156.80 | 63.17 |
| 010 | Carifree® Pro Gel 5000 | 297 | 318 | 310 | 287 | 294 | 301.20 | 12.56 | 76 | 88 | 83 | 85 | 81 | 82.60 | 4.51 | -218.60 | 231 | 249 | 200 | 227 | 267 | 234.80 | 25.12 | 152.20 | 69.62 |
| 004 | Carifree® Pro Gel 5000 | 329 | 332 | 323 | 307 | 335 | 325.20 | 11.10 | 110 | 107 | 98 | 106 | 123 | 108.80 | 9.09 | -216.40 | 245 | 241 | 227 | 265 | 249 | 245.40 | 13.74 | 136.60 | 63.12 |
| 007 | Carifree® Pro Gel 5000 | 320 | 320 | 341 | 341 | 310 | 326.40 | 13.94 | 110 | 131 | 119 | 146 | 110 | 123.20 | 15.39 | -203.20 | 203 | 251 | 201 | 241 | 265 | 232.20 | 28.87 | 109.00 | 53.64 |
| 017 | Carifree® Pro Gel 5000 | 335 | 335 | 335 | 347 | 360 | 342.40 | 11.13 | 161 | 147 | 142 | 149 | 132 | 146.20 | 10.57 | -196.20 | 252 | 245 | 234 | 260 | 273 | 252.80 | 14.79 | 106.60 | 54.33 |
| 020 | Carifree® Pro Gel 5000 | 312 | 332 | 335 | 307 | 299 | 317.00 | 15.80 | 126 | 110 | 129 | 152 | 131 | 129.60 | 15.01 | -187.40 | 245 | 216 | 217 | 249 | 225 | 230.40 | 15.61 | 100.80 | 53.79 |
| 023 | Carifree® Pro Gel 5000 | 312 | 364 | 354 | 332 | 312 | 334.80 | 23.82 | 181 | 150 | 152 | 140 | 182 | 161.00 | 19.26 | -173.80 | 275 | 310 | 287 | 326 | 239 | 287.40 | 33.53 | 126.40 | 72.73 |
| 011 | Carifree® Pro Gel 5000 | 318 | 318 | 307 | 315 | 294 | 310.40 | 10.21 | 139 | 158 | 149 | 140 | 132 | 143.60 | 10.06 | -166.80 | 269 | 243 | 247 | 258 | 249 | 253.20 | 10.40 | 109.60 | 65.71 |
| 003 | Carifree® Pro Gel 5000 | 320 | 312 | 323 | 320 | 302 | 315.40 | 8.53 | 157 | 163 | 156 | 151 | 162 | 157.80 | 4.87 | -157.60 | 251 | 271 | 267 | 278 | 267 | 266.80 | 9.91 | 109.00 | 69.16 |
| 015 | Carifree® Pro Gel 5000 | 271 | 267 | 263 | 265 | 273 | 267.80 | 4.15 | 132 | 129 | 145 | 129 | 112 | 129.40 | 11.76 | -138.40 | 231 | 208 | 236 | 227 | 192 | 218.80 | 18.35 | 89.40 | 64.60 |
| | | | | | | | | | | | | | | **Average** | | **-190.66** | | | | | | **Average** | | **119.64** | **62.99** |
| | | | | | | | | | | | | | | **StnDev** | | **32.71** | | | | | | **StnDev** | | **22.46** | **6.94** |
| 016 | DI Water | 304 | 285 | 310 | 299 | 323 | 304.20 | 13.99 | 54 | 69 | 61 | 62 | 75 | 64.20 | 8.04 | -240.00 | 197 | 179 | 183 | 150 | 182 | 178.20 | 17.22 | 114.00 | 47.50 |
| 005 | DI Water | 312 | 344 | 329 | 323 | 304 | 322.40 | 15.47 | 98 | 109 | 110 | 98 | 101 | 103.20 | 5.89 | -219.20 | 211 | 204 | 205 | 192 | 194 | 201.20 | 7.98 | 98.00 | 44.71 |
| 013 | DI Water | 292 | 287 | 307 | 312 | 318 | 303.20 | 13.22 | 85 | 94 | 96 | 96 | 83 | 90.80 | 6.30 | -212.40 | 194 | 194 | 219 | 198 | 185 | 198.00 | 12.67 | 107.20 | 50.47 |
| 021 | DI Water | 271 | 299 | 302 | 271 | 280 | 284.60 | 15.01 | 74 | 77 | 64 | 80 | 75 | 74.00 | 6.04 | -210.60 | 157 | 168 | 179 | 184 | 200 | 177.60 | 16.29 | 103.60 | 49.19 |
| 001 | DI Water | 294 | 302 | 312 | 332 | 312 | 310.40 | 14.24 | 112 | 127 | 131 | 94 | 108 | 114.40 | 14.98 | -196.00 | 181 | 203 | 239 | 204 | 191 | 203.60 | 21.93 | 89.20 | 45.51 |
| 024 | DI Water | 302 | 302 | 315 | 307 | 310 | 307.20 | 5.54 | 101 | 116 | 125 | 105 | 128 | 115.00 | 11.90 | -192.20 | 188 | 182 | 196 | 179 | 182 | 185.40 | 6.77 | 70.40 | 36.63 |
| 012 | DI Water | 299 | 323 | 297 | 320 | 302 | 308.20 | 12.32 | 133 | 145 | 150 | 137 | 126 | 138.20 | 9.52 | -170.00 | 217 | 203 | 216 | 178 | 210 | 204.80 | 15.99 | 66.60 | 39.18 |
| 006 | DI Water | 323 | 312 | 292 | 320 | 304 | 310.20 | 12.58 | 154 | 124 | 137 | 141 | 156 | 142.40 | 13.13 | -167.80 | 192 | 182 | 141 | 145 | 162 | 164.40 | 22.37 | 22.00 | 13.11 |
| 008 | DI Water | 357 | 354 | 354 | 344 | 347 | 351.20 | 5.45 | 234 | 219 | 236 | 185 | 182 | 211.20 | 26.15 | -140.00 | 229 | 217 | 239 | 273 | 232 | 238.00 | 21.12 | 26.80 | 19.14 |
| 014 | DI Water | 287 | 315 | 294 | 320 | 323 | 307.80 | 16.24 | 166 | 168 | 177 | 150 | 178 | 167.80 | 11.28 | -140.00 | 216 | 198 | 221 | 205 | 216 | 211.20 | 9.42 | 43.40 | 31.00 |
| | | | | | | | | | | | | | | **Average** | | **-188.82** | | | | | | **Average** | | **74.12** | **37.64** |
| | | | | | | | | | | | | | | **StnDev** | | **33.66** | | | | | | **StnDev** | | **33.86** | **12.90** |

**Appendix 2: Statistical Analysis**

Descriptive Statistics: % SMHR
Statistics

| Variable | Treatment Group | N | Mean | StDev | Minimum | Median | Maximum |
| --- | --- | --- | --- | --- | --- | --- | --- |
| % SMHR | Carifree® Pro Gel 5000 | 10 | 62.99 | 6.94 | 53.64 | 63.89 | 72.73 |
| | DI Water | 10 | 37.64 | 12.90 | 13.11 | 41.94 | 50.47 |

Two-Sample T-Test and CI: % SMHR_Carifree® Pro Gel ... R_DI Water
Method

$\mu_1$: mean of % SMHR_Carifree® Pro Gel 5000

$\mu_2$: mean of % SMHR_DI Water

Difference: $\mu_1 - \mu_2$

*Equal variances are not assumed for this analysis.*

Descriptive Statistics

| Sample | N | Mean | StDev | SE Mean |
| --- | --- | --- | --- | --- |
| % SMHR_Carifree® Pro Gel 5000 | 10 | 62.99 | 6.94 | 2.2 |
| % SMHR_DI Water | 10 | 37.6 | 12.9 | 4.1 |

Estimation for Difference

| Difference | 95% CI for Difference |
| --- | --- |
| 25.34 | (15.33, 35.35) |

Test

| | |
| --- | --- |
| Null hypothesis | $H_o$: $\mu_1 - \mu_2 = 0$ |
| Alternative hypothesis | $H_1$: $\mu_1 - \mu_2 \neq 0$ |

| T-Value | DF | P-Value |
| --- | --- | --- |
| 5.47 | 13 | 0.000 |

**Report signature:**

I declare that this report constitutes a true and faithful account of the procedures adopted and the results obtained in the performance of this study.

Gavin Thomas .....

(Laboratory Manager, Intertek CRS) Date..... 13th Oct 2025


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