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Title: Remineralization of natural early caries lesions in vitro by P_{11}-4 monitored with The Canary System® and The Canary Lab

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Introduction:

The recent Global Burden of Disease study showed that untreated caries in the permanent dentition ranked first out of 291 diseases, affecting more than 35% of the global population, while untreated caries in deciduous teeth ranked 10th (9% of the global population)[1]. Employing methods and agents that are effective in monitoring the progression and the promotion of remineralization of early caries lesions will be an important part in the future armory of clinicians. There is currently no consensus view as to how the white spot lesion, indicative of early caries, should be treated [2]. Clinicians can: 1) monitor the lesion to determine whether or not it is getting bigger (but this requires reliable and non-invasive tools to do so), then excavate and fill if necessary; 2) apply fluoride treatments, then proceed as in (1) or 3) place a small restoration - a filling- based upon clinical judgment. There is a finite lifespan to all restorative procedures so trying to remineralize lesions may help to avoid the initial placement and then replacement of restorations.

The white spot (opacity) that is characteristic of early caries is due to porosity within the underlying enamel caused by acid dissolution (demineralisation) of the existing hydroxyapatite crystal structure. In order to enable regeneration or remineralisation of this subsurface lesion, a scaffold is needed to template hydroxyapatite formation and help to align the crystals analogous to the role of matrix proteins during enamel formation [3]. This scaffold needs to be able to infiltrate into the demineralised tissue. As such, the scaffold should ideally form spontaneously within the lesion body.

Previous experiments have shown that monomeric self-assembling peptide P_{11}-4 diffuses into the subsurface of early caries lesions and self-assembles into 3D fibrillar scaffolds in response to local conditions of high ionic strength and acidic pH within the lesion body [4]. The assembled P_{11}-4 scaffold is capable of templating *de novo* hydroxyapatite crystal nucleation and supports mineral crystal growth in a process of biomimetic mineralization, regenerating the enamel analogous to the enamel matrix during enamel formation [4]. In previous studies,

P_{11}-4 was shown to promote subsurface mineralisation in the presence of saliva both *in vitro* and *in vivo* [4-7]. P_{11}-4 is now the basis of a product, Curodont™ Repair (Credentis AG, Switzerland) which is now approved and available for clinical use in the treatment of early enamel lesions.

An adjunct method capable of detecting and quantitatively monitoring the progression of early caries would be of value in the prevention, treatment and development of new therapies for dental caries. In recent years, two instruments aimed at addressing these needs have been developed and commercialized by Quantum Dental Technologies Inc (Canada). The Canary System® has been used as a clinical aid for the non-invasive early detection and monitoring of caries lesions[8-10], while the Canary Lab is a non-destructive imaging research instrument for detecting and monitoring dental caries and acid erosion *in vitro* [11]. Both The Canary System and Canary Lab directly assess the status of the tooth crystal structure by using energy conversion technology termed photothermal radiometry and luminescence (PTR-LUM). The PTR-LUM detection technique has been validated by previous *in vitro* and *in vivo* studies and has been shown to have the ability to detect and monitor demineralization and remineralization of early caries lesions [9, 12-17]. Intensity-modulated laser light at a fixed frequency is shone on to the tooth surface and the light is converted into both heat (PTR) and light of longer wavelength (LUM), thereby generating thermal infrared and optical emissions at the same frequency [9]. At the same time, simultaneously captured back-scattered laser light carries information about the near-surface-structure-related optical properties of scanned teeth. The Canary System measures four signals from an area as wide as 1.5 mm and as deep as 5 mm: (i) strength of the converted heat (PTR amplitude; PTR-A); (ii) time delay of the converted heat to reach the surface conductively (PTR phase; PTR-P); (iii) strength of the converted luminescent and back-scattered laser light (LUM amplitude; LUM-A) and (iv) time delay of the converted luminescent light (LUM phase; LUM-P). An algorithm combines these four signals to generate the “Canary Number” (CN), which reflects the status of the tooth crystal structure or

mineral architecture. The CN increases as early mineral loss from the tooth (incipient caries) causes small changes in the ultrastructure, creating a more porous, less mineral-dense environment. Correspondingly, if remineralization of the lesion progresses, the CN decreases as the lesion becomes less porous and exhibits a denser mineral environment.

Our hypothesis was that a single treatment with P_{11}-4 could promote the regeneration of natural early caries lesions in human teeth over time. Thus the objective of the present study was to determine the ability of the P_{11}-4 to promote the remineralization of natural initial caries lesions. The remineralization process was monitored by The Canary System® and Canary Lab, which have been established as useful tools for quantitative monitoring of the remineralization of early caries lesions of enamel following administration of a therapeutic non-invasive intervention.

Methods:

Study Design. Tooth selection: Following approval (IRB Approval #: HSC20080233N) by the Institutional Review Board of the University of Texas Health Science Center at San Antonio (UTHSCSA), freshly extracted permanent human teeth (molars and premolars) obtained from the clinics of the UTHSCSA dental school were collected and examined. Teeth were selected on the basis that they included smooth surfaces without debris, stains, plaques, restorations, sealants or cavitations, but with visually sound enamel and early caries lesions, identified as white or brown spots. All tooth samples were stored and periodically refreshed in deionized distilled water to avoid dehydration.

Baseline Readings: Baseline CN readings were obtained by an experienced operator scanning all of the selected enamel sites (both sound and caries) on all of the teeth using The Canary System (Model: L-CS-CO-001) and The Canary Lab (Model: L-CL-CO-001) according to manufacturer's instructions (Quantum Dental Technologies Inc; Toronto, Canada). The smooth surfaces were photographed and the positions of the examined sites were clearly marked on each photograph to ensure measurements at identical sites were performed in follow-up measurements. Teeth were randomly assigned to three experimental groups: 1) Treatment Group, treated with P_{11}-4 (Curodont™ Repair, Credentis AG, Windisch, Switzerland) according to instructions given by the manufacturer (20 second exposure to NaClO (3%); 20 second rinsing with H_2O; 20 second exposure to dental Etching Gel (containing 37% phosphoric acid); 20 second rinsing with H_2O); 5 min contact time of P11-4 solution; 2) Control Group, sites remained untreated throughout the experiment, and 3) Placebo Group, treated with an identical formulation and cleaning procedure to the Treatment Group but without P_{11}-4 applied as described in (1) above. After application of P_{11}-4 and placebo by a clinician blinded to the sample identities, all examination sites were re-scanned in a blinded-fashion with The Canary System and Canary Lab. These scans were designated as Day 0. All tooth samples were then stored and refreshed daily in 10-15mL artificial saliva solution in 50mL Falcon Tubes ($MgCl_2.6H_2O$ (0.148 mmol/L), K_2HPO_4 (4.59 mmol/L), KH_2PO_4 (2.38 mmol/L), KCL (8.39 mmol/L), calcium lactate (1.76 mmol/L),

fluoride (0.05 ppm) as NaF, sodium carboxymethylcellulose (2.25 mmol/L), methyl-4-hydroxybenzoate (13.14 mmol/L), pH adjusted to 7.2 using KOH [18] for a total of 50 days. Sites were scanned with The Canary System and Canary Lab after 7, 14, 30, and 50 days.

The Canary System assessment. A total of 34 visibly sound and 28 visibly carious smooth surface sites were scanned using The Canary System comprising of: Control Group, 10 sound and 9 carious sites; Treatment Group, 14 sound and 10 carious sites and Placebo Group, 10 sound and 9 carious sites. The Canary System® was calibrated prior to use in accordance with the manufacturer's operating instructions to obtain readings from the smooth surface sites at baseline and Days 0, 7, 14, 30, and 50. Each tooth sample was removed from the remineralization solution, rinsed thoroughly with clean deionized distilled water for 20 seconds, and air-dried for five seconds before measurement. Three measurements were taken for each site and the average CN was calculated at each time point.

The Canary Lab assessment. A region measuring ≤6 mm x 6 mm was identified on each smooth surface site of interest (measurement resolution = 250 µm where each measurement site had 25 x 25 pixels) and was scanned using the Canary Lab. A total of 305 visibly sound sites and 936 carious sites were scanned comprising of: Control Group, 106 sound and 243 carious sites; Treatment Group, 75 sound and 386 carious sites and Placebo Group, 124 sound and 307 carious sites. The Canary Lab was calibrated prior to use in accordance with the manufacturer's operating instructions to obtain readings from the smooth surface at baseline and Days 0, 7, 14, 30, and 50. Tooth samples were prepared for scanning similar to what was performed, as above, for The Canary System assessments.

Histological Validation. After all the measurements were completed at Day 50, polarized light microscopy (PLM) was performed by a blinded operator who is an expert in cariology to confirm the presence or absence of a clinically significant caries lesion at each examination site. A tooth section was cut at a sagittal plane from the center of each site, and was examined histologically under the polarizing light microscope (Model BH-2, Olympus, Japan). For each section, the image was captured using a digital camera (Axio Cam ICc 1,

Zeiss, Germany) connected to the microscope. These images were then used to measure the depth (μm) of each lesion using the PLM AxioVs40 4.8.1.0 Software (Carl Zeiss, Germany).

Data and Statistical Analyses. For The Canary System, a time series plot was produced to show mean CNs and changes in the CNs for the following six categories of samples as functions of time after treatment: 1) Untreated sound sites; 2) Untreated carious sites; 3) Treated sound sites; 4) Treated carious sites; 5) Placebo sound sites; and 6) Placebo carious sites. Statistically significant differences in CN compared to baseline were determined by Related-Samples Wilcoxon Signed Rank Test ($p < 0.05$). Statistically significant differences in the changes of the CN between different categories were determined by Student t-test ($p < 0.05$). For statistical analyses, the CN of each site was identified as “sound” or “carious”, where CN readings were ranked according to the Canary Scale: 0 to 20 = sound; 21 to 100 = carious.

Results:

Representative photographs of selected scanning regions for The Canary System (Panels A and B), PLM images (Panel C), and Canary Lab images (Panel D)for representative samples from each of the Control (Figure 1), Treated (Figure 2) and Placebo (Figure 3) groups are shown.

Over the course of the 50-day study, mean CNs derived from the CS from all sound sites among all groups remained in the "healthy" zone (CN ≤20). CNs from carious sites in the Placebo and Untreated groups remained in the "decayed" zone (CN >20). However, by Day 30, the average CN began to significantly decrease (p=0.03) for carious samples treated with P_{11}-4 suggesting enamel regeneration. This significant (p = 0.008) decrease in CN continued through to Day 50 at the end of the study (p = 0.008). Overall, carious sites treated with P_{11}-4 decreased in CN from mean 44±3.8 at baseline to mean 24±4.9 at day 50 (Figure 4). By Day 50, there were no significant differences in CNs between carious samples treated with P_{11}-4 and untreated sound samples, P_{11}-4-treated sound samples, or placebo sound samples, suggesting that carious samples treated with P_{11}-4 resulted in almost full regeneration to a sound sample state.

When examining the PTR-LUM signals behind CNs derived from the CS, significant differences were found between baseline and Day 50 samples treated with P_{11}-4 ($p < 0.05$). PTR-A and PTR-P responses (which are directly proportionate to CN) decreased between baseline (84.4±20.0 and 34.1± 2.0) and Day 50 (34.6±14.8 and 28.7±1.9) after treatment with P_{11}-4, respectively. LUM-Amplitude response (which is indirectly proportional to CN) increased between baseline (46.5±1.0) and Day 50 (10.4.3±13.8) after treatment with P_{11}-4 (p =0.003). No significant differences were found in PTR-LUM signals between days among the study groups (data not shown).

Canary Lab images (Panels D, Figures 1-3) clearly illustrated and provided quantification for the changes in the carious sites associated with P_{11}-4 treatment. The individual images and values of PTR-A, PTR-P and LUM-A values, and hence CN, all indicated remineralization of the lesions. Canary Lab permitted objective, systematic measurements of the 6 mm x 6 mm regions from each tooth surface acquired in a series of 0.25 mm steps. (The Canary System measures one single spot on the tooth that is 1.5 mm wide by 5 mm deep for each step). Canary Lab images of carious sites were captured at baseline and again on day 50 after treatment P_{11}-4. Overall, the mean CN from the region-of-interest (6 mm x 6 mm) significantly decreased ($p < 0.05$) between baseline (65±7.6) and after the 50th day of treatment (45±2.9) with P_{11}-4 (data not shown).

Overall, CN and PTR-LUM values obtained with The Canary System and the Canary Lab indicated a significant regression ($p < 0.05$) of natural caries lesions after a single application of P_{11}-4 compared with Control and Placebo groups. When examining the delta (relative change in CN unit from baseline) between time points, changes in CN were evident when comparisons were made between Untreated vs P_{11}-4-Treated and Placebo vs P_{11}-4-Treated groups starting at Day 7 with the largest Delta at Days 30 and 50 ($p<0.001$). Small but significant changes of CNs derived from scans with The Canary System were only observed at individual time points for the sound enamel sites and did not show a clear trend (Table 1).

PLM was employed to validate the presence or absence of caries at scanned sites (Table 2) and was found to be 100% aligned with the visual identification of carious sites from samples in all three groups. PLM also had a very strong correlation with the visual identification of sound sites in the Untreated (10/10) and Treated Groups (13/14). However, in the Placebo Group, PLM indicated that 6/10 sites were correctly identified as sound sites, and 4/10 sites had some degree of demineralisation with an average lesion depth of 272±136µm. It appeared that the amount and size of the demineralized area (as indicated by PLM) in these sites in the Placebo group, that had been visually identified as sound sites, was so small that

readings from the Canary also did not detect them as such (i.e. CN >20). In contrast, the degree of demineralization of PLM-validated carious sites in all three groups were clearly more advanced and could be seen visually as white spots, with average lesion depths for P_{11}-4-Treated, Control, and Placebo groups of 732±424 µm, 527±212 µm, and 447±191 µm, respectively. Therefore, despite the fact that the visually sound sites allocated to the Placebo-Sound group did appear to have small areas of caries on the PLM, this did not impact on the overall results of the study because these sites occupied a very small area and were clinically identified as sound according to The Canary System when the Canary laser is pulsed at a frequency of 2 Hz.

Discussion:

In the present era of preventive and minimal invasive dentistry, the benefit of early detection and remineralization or arrest of caries lesions cannot be overemphasized. In addition to saving the cost of restorative treatment, it would prevent invasive intervention that commits the tooth into a circle of treatment and re-treatment, known as the 'death spiral of restorations' [19]. Promotion of the natural repair processes by influencing etiological factors such as oral hygiene education, dietary control, and local fluoridation [20] requires patient compliance to be successful. A "natural" repair process, such as a regenerative treatment of caries, which does not rely upon the patient's compliance, is becoming a focus in dentistry [21]. P_{11}-4 has been described as a biomimetic mineralisation agent and has shown promising results in *in vitro* [4, 22] and *in vivo* studies [5, 7, 23]. P_{11}-4 assembles to form hierarchical 3D fibrillar networks under physiological conditions such as what would be anticipated within caries lesions [24-26]. The peptide was designed such that on assembly into fibrils, surface charge domains arising from glutamate residues arrayed along the fibrillar surface would provide potential nucleating sites for hydroxyapatite crystals and the fibrillar matrix as a whole would support secondary crystal growth as a Guided Enamel Regeneration. In

support of this hypothesis, i*n vitro* data has shown a net mineral gain after application of P_{11}-4 to artificial caries lesions after 5d of oscillating pH [4].

In the present study, the effects of Curodont™ Repair, which contains P_{11}-4, on the remineralisation of natural caries lesions was monitored and quantified using The Canary System and Canary Lab. The results showed a significant decrease in the PTR-LUM values (i.e. Canary Number) after one application of P_{11}-4 compared to controls, where no significant decreases in CNs were observed for early caries left untreated or treated with a sham application including the preparation of the lesion site with NaOCl and Etching gel (30% H_3PO_4). The controls showed that spontaneous remineralisation of the natural lesions on human teeth with artificial saliva was not possible under the laboratory conditions used here. In addition, treatment of the lesion surfaces by etching did appear to have initiated measurable remineralisation. Each tooth used in the study provided its own internal control as sound enamel sites were chosen on each tooth for all three treatment groups to provide a comparison with the carious sites. For these sites, CNs remained constant, as expected, throughout the treatment period. For the carious lesions treated with P_{11}-4 both The Canary System and the Canary Lab showed the same trend in lesion remineralisation. Canary Numbers from The Canary System showed the greatest decrease by Day 30 after treatment; however, the greatest decrease in CN from The Canary Lab did not occur until the Day 50 measurement. This may be explained by differences in the ways in which these two systems measure samples. For each carious site, a one point (~1.0 mm $\pm$ 0.2 mm effective beam spot at the tip contact point) scan was performed using The Canary System and the average CN was taken from an average of 3 repeat point scans. In the case of Canary Lab, a carious region ≤6 x 6 mm which was divided into 25 x 25 pixels was examined. Each pixel (or site; 250 µm x 250 µm) was scanned by Canary Lab and a CN was obtained. The average CN was taken from the CNs obtained from all sites (25 x 25) within the examined carious region. Therefore, Canary Lab examined a much larger number of point scans per carious region than The Canary System, which only measured a relatively localized scan area per carious

site. This most likely resulted in average CNs that appeared to show a more gradual decrease over time. Previous studies showed good correlation between the PTR-LUM technology and artificially induced enamel demineralization (mineral losses) with transverse microradiography (TMR) and microcomputer tomography (μ-CT) results [27, 28]. For future studies involving The Canary System, TMR and μ-CT, which measure not only lesion depth but also mineral losses, may be additional methods to examine correlation between CNs and mineral losses.

We elected to use a non-pH cycling experimental strategy for this work as our main objective was to investigate the ability of P_{11}-4 to promote the remineralization of natural caries lesions. We were therefore unable to determine the effect of cycling and lower pH on any new mineral deposited within the lesions but earlier work using artificial caries-like lesions under conditions of oscillating pH clearly demonstrated an inhibition of demineralisation following application of P_{11}-4 and a net gain of mineral over the course of the 5d experiment, supporting the findings reported here[4].

Conclusion:

Based on measurements obtained from natural lesions of human enamel, one treatment with P_{11}-4 enabled enamel regeneration, presumably by promoting *de novo* remineralisation of the early caries lesions as detected and monitored by The Canary System and the Canary Lab.

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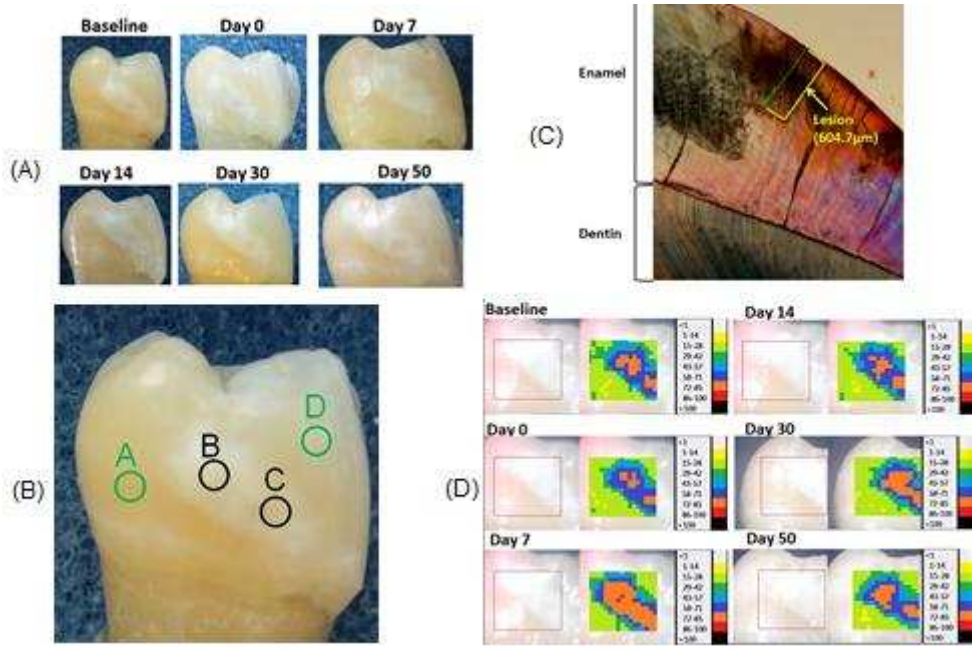

Figure 1. Representative Control Group (untreated) sample. (A) Time-course photographs of tooth surface examined. Visually, the tooth surface did not change in appearance over the treatment period. (B) Sites examined by scanning. Sound sites are outlined by green circles, and carious sites are outlined by black circles. (C) Polarized light microscopy image of examination site B at Day 50. The lesion was 605 µm in depth. (D) Time-course of Canary Lab images of the tooth were comparable throughout treatment period.

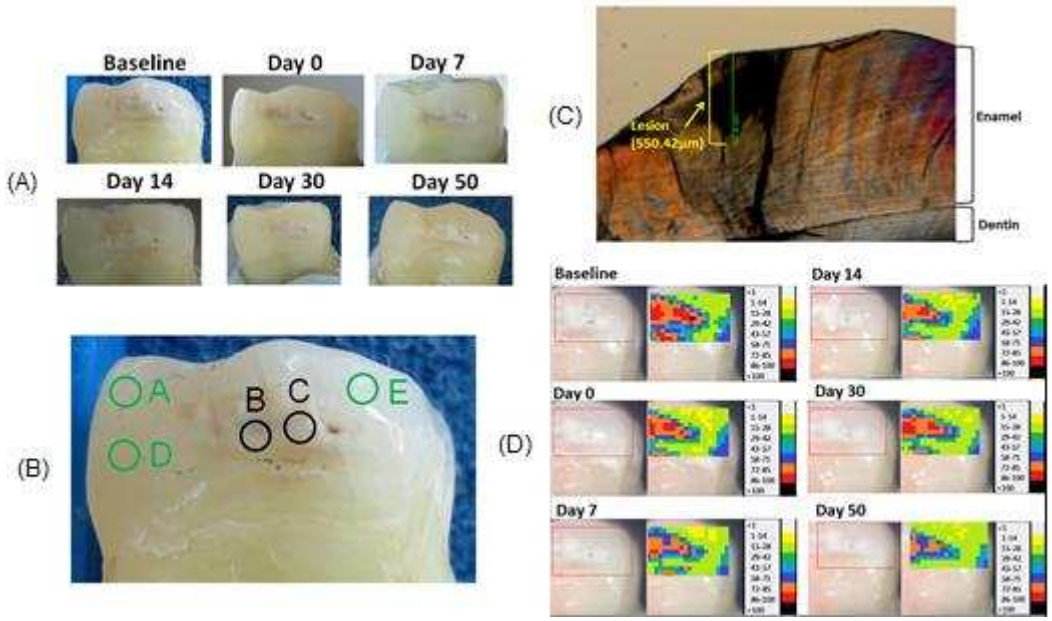

Figure 2. Representative Treatment Group tooth sample treated with P_{11}-4. (A) Time-course photographs of tooth surface examined. Visually, the tooth surface did not change in appearance over the treatment period. (B) Sites examined by scanning. Sound sites are outlined by green circles, and carious sites are outlined by black circles. (C) Polarized light microscopy image of examination site B at Day 50. The lesion was 550 µm in depth. (D) Time-course of Canary Lab images of tooth. Remineralization of the treated tooth structures were reflected in the Canary Lab images by the fact that colors representing higher Canary Numbers (orange/red) were replaced with colors representing lower Canary Numbers (yellow/green).

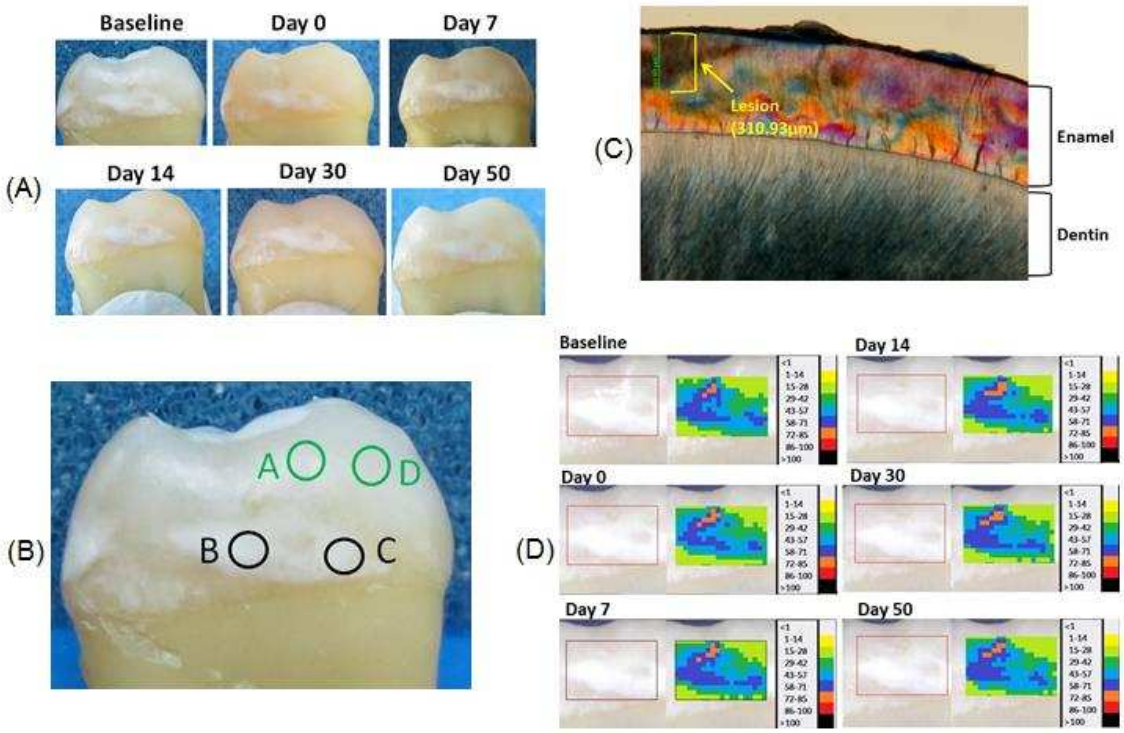

Figure 3. Representative Placebo Group sample. Placebo Group samples were treated with an identical formulation to the test group but without P_{11}-4 after preparation of the site. (A) Time-course photographs of tooth surface examined. Visually, the tooth surface did not change in appearance over the treatment period. (B) Sites examined by scanning. Sound sites are outlined by green circles, and carious sites are outlined by black circles. (C) Polarized light microscopy image of examination site at Day 50. The lesion was 311 µm in depth. (D) Canary Lab images of tooth were comparable throughout treatment period.

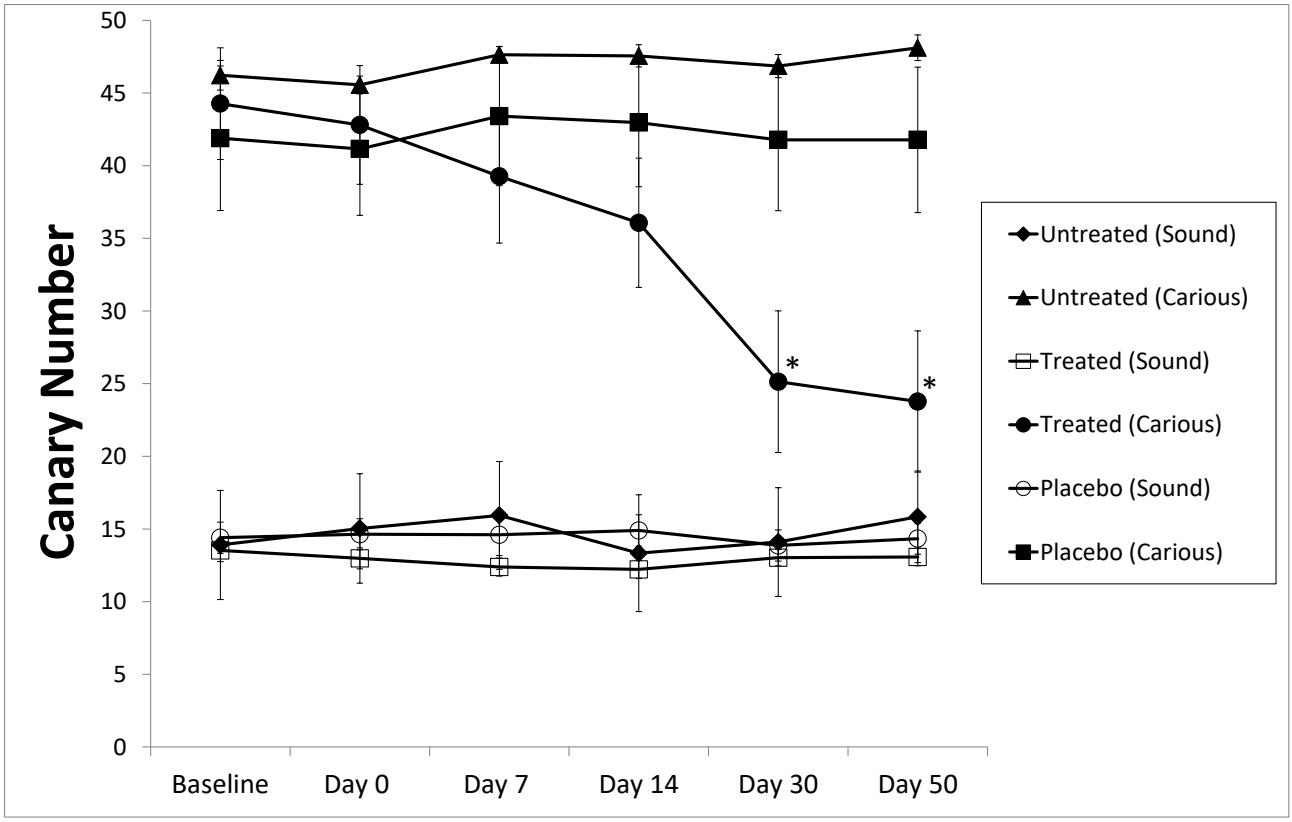

Figure 4. Mean Canary Numbers from each group plotted over time. Untreated Group: no treatment; Treated Group: samples treated with P_{11}-4; Placebo Group: samples were treated with an identical formulation as the test group but without P_{11}-4 after preparation of the site. CNs from all sound sites among all groups remained in the healthy zone (≤20). CNs from carious sites in the Placebo and Untreated groups remained in the decayed zone (>20). CNs from carious sites treated with P_{11}-4 exhibit a significant and sustained decrease starting at day 30. Asterisks (*) represent statistically significant differences in Canary Numbers compared to baseline. Statistically significant differences were determined by Related-Samples Wilcoxon Signed Rank Test ($p < 0.05$).

	BASELINE	D0	D7	D14	D30	D50
Caries-Sites						
UNTREATED VS P_{11}-4	0.0	-0.8	***-6.4***	***-9.5***	***-19.8***	***-22.4***
UNTREATED VS PLACEBO	0.0	-0.1	0.1	-0.3	-0.7	-2.0
PLACEBO VS P_{11}-4	0.0	-0.7	***-6.5***	***-9.3***	***-19.0***	***-20.4***
Sound Enamel-Sites						
UNTREATED VS P_{11}-4	0.0	-1.7	**-3.2**	-0.7	-0.7	**-2.4**
UNTREATED VS PLACEBO	0.0	-0.9	**-1.8**	1.1	-0.7	**-2.0**
PLACEBO VS P_{11}-4	0.0	-0.8	-1.3	-1.8	0.0	-0.4

Table 1. Changes of the Canary Number within one group compared to other groups. Statistically significant values ($p<0.05$) in bold and highly significant values ($p<0.001$) in bold and italic.

Group	**Visually Sound Sites**		**Visually Carious Sites**	
	Number of Sites Confirmed by PLM	Average Lesion Depth (µm) of Sites Identified as Carious	Number of Sites Confirmed by PLM	Average Lesion Depth (µm)
CONTROL (UNTREATED)	10/10 (100%)	0	9/9 (100%)	527 ± 212
PLACEBO	6/10 (60%)	272 ± 136	9/9 (100%)	468 ± 215
P_{11}-4-TREATED	13/14 (93%)	250	10/10 (100%)	732 ± 424

Table 2. Polarized light microscopy (PLM) results of tooth samples. PLM was employed to validate the presence or absence of caries at scanned sites.