A NOVEL APPROACH TO AESTHETICALLY TREAT ARRESTED CARIES LESIONS

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DEDICATION

First I thank Allah, for giving me the strength, courage, guidance and blessing me to accomplish this dissertation.

I dedicate this thesis to my beloved parents (Dr. Sultan and Khairia), my kids (Ibrahim and Reema), sisters (Nadia and Samiah) and brothers (Abdulaziz, Hisham and Hussam) a great source of motivation, inspiration, and support. A special thanks to my dearest friend Hadeel Ayoub who was there for me the whole time. May God bless them all.

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Sarah Sultan A. Alangari

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In this thesis, we proposed and investigated the efficacy and safety of dental bleaching as a non-invasive aesthetic treatment option for stained arrested caries lesions (s-ACLs). Chapter 1 reports the suitability of this approach in extracted human teeth, as well as in a selected clinical case. Visual improvement in the color lighteness of the s-ACLs was observed and reported in photographs. In order to systematically study the impact of dental bleaching on the s-ACLs, we developed in vitro models simulating the development of metallic and non-metallic s-ACLs (Chapter 2). Human dental specimens were submitted to incipient caries-like lesion formation, followed by a 5-day cycling protocol based on remineralization and staining episodes. The created lesions were then bleached (simulating in-office/40% hydrogen peroxide). Color change was measured spectrophotometrically at baseline, after lesion creation, staining/remineralization cycling and bleaching; while mineral loss and lesion depth were quantified by transversal microradiography after staining/remineralization cycling. Metallic s-ACLs were darker, more remineralized and more difficult to bleach, compared to the non-metallic ones ($p<0.05$). In Chapter 3, we tested the efficacy and safety of different dental bleaching systems (simulating at-home/15% carbamide peroxide and in-office/40% hydrogen peroxide) using the in vitro models previously developed. Similar methods and outcomes were used, with the addition of demineralization after bleaching to simulate and test changes in caries susceptibility. At-home bleaching showed greater efficacy in color

improvement compared to in-office ($p<0.05$), but also increased susceptibility to further demineralization ($p<0.05$), regardless of the type of stain. Overall, bleached non-metallic s-ACLs were more susceptible to demineralization compared to metallic ones ($p<0.05$). Within the limited laboratory testing conditions, we concluded that dental bleaching can improve the aesthetics of s-ACLs, with efficacy being dependent on the nature of the stain. At-home bleaching presented greater efficacy, but also raised some potential safety concerns, which should be further investigated in clinical conditions. In-office bleaching protocol showed to be an effective and safe procedure for the aesthetic treatment of stained arrested caries lesions.

Anderson T. Hara, DDS, MS, PhD, Chair

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GENERAL INTRODUCTION

During the last decades, aesthetic dental treatments have been highly prevalent in clinical practice [1]. With the improvements in diagnostic tools and dental materials conservative therapeutic approaches have been established. This has directed the clinician's attention to minimal invasive and conservative treatment strategies [2]. Active caries lesions can be arrested by preventive measures, including different remineralizing techniques and materials such as fluoride [3-5]. However, during remineralization stains are likely to incorporate within the caries lesion, which may potentially result in a noticeable and unaesthetic dark discolored lesion [6, 7].

The standard aesthetic treatment to manage stained arrested caries lesions (s-ACLs) usually involves surgical intervention [1, 8, 9]. This is concerning, as patients nowadays have a better perception of aesthetic dental care, demanding treatments with optimum health and function along with improved aesthetics [1, 10]. Therefore, a sensible strategy is to start with the least invasive approach and progress to the most invasive one, as needed.

Bleaching has been one of the most common non-invasive treatments for discolored teeth since it has been shown to be effective, predictable and economical [11]. In this respect, it eliminates discolorations, minimizes unnecessary surgical treatment and reduces the possibility of iatrogeny. Therefore, we consider it to be an advantageous option to treat darkly s-ACLs.

There is a need, however, to establish and test this novel approach before adopting it to treat s-ACLs clinically. In this thesis we aimed to evaluate the efficacy and safety of the proposed approach, by assessing its effects on the physical properties and demineralization susceptibility of s-ACLs artificially created *in vitro*. Our hypothesis was that bleaching systems would improve the esthetics of s-ACLs. The hypothesis has been formulated on the basis of preliminary laboratory/clinical data, showing that carbamide peroxide and hydrogen peroxide gels can substantially improve the aesthetics of ACLs. The hypothesis was tested by pursuing three specific aims, each related to each of the three chapters of this thesis.

- Specific Aim 1: to report and present the efficacy of a conservative approach for the treatment of s-ACL, based on the use of dental bleaching systems on both naturally stained ACL in extracted teeth as well as in a clinical case (Chapter 1).

- Specific Aim 2: to develop laboratory models, utilizing a modified pH-cycling model (based on demineralization/remineralization episodes) to create remineralized stained caries-like lesions, based on metallic/non-metallic stains, and to test the efficacy of bleaching on the aesthetic treatment of the created lesions by evaluating color change and mineral content (Chapter 2).

- Specific Aim 3: to evaluate the efficacy of different bleaching systems on artifically created remineralized-stained caries-like lesions utilizing different

stain types and to assess the susceptibility of the treated lesions to further demineralization by evaluating color change and mineral content (Chapter 3).

CHAPTER ONE

A Conservative Approach to Aesthetically Treat Stained Arrested Caries Lesions

Summary

Aesthetic treatment of stained arrested caries lesions (ACLs) has mostly been done using invasive restorative techniques. The aim of this paper was to propose and report the efficacy of a conservative approach based on dental bleaching to esthetically treat these lesions, both experimentally (extracted teeth) and clinically. In part one, ten extracted human teeth with stained ACLs in either pits and fissures or smooth surface were selected and treated with 15 % carbamide peroxide gel, 4 h per day, for a total of six days. Part two reports a clinical case of pit and fissure stained ACLs in four posterior teeth, which were treated with 40 % hydrogen peroxide in-office bleaching. Digital photographs were taken in both parts to document the efficacy of the treatment. The lesions showed noticeable increase in color lightness indicating the efficacy and suitability of the proposed approach. By using the conservative clinical technique presented, the aesthetics of most stained ACLs could be improved, eliminating the need of invasive restorative treatments.

Introduction

During the last decades, aesthetic dental care has been one of the most prevalent treatments in clinical practice. Patients nowadays demand treatments with not only optimum health and function but also improved aesthetics.[1] In this regard, advances in dental materials and diagnostic technologies have improved leading to conservative treatment strategies, emphasizing minimally invasive and cost-effective treatments.[2] Incipient caries lesions can be conservatively treated using different remineralizing agents. Arrested caries lesions (ACL), on the other hand, are remineralized lesions that are no longer active, presenting highly mineralized surfaces,[3] along with a frequent and undesirable dark discoloration, due to pigment incorporation during the remineralization process. This scenario may cause some patients to psychologically refuse the unaesthetic appearance of the dark ACL,[1] commonly managed by surgical intervention.[4]

To satisfy the aesthetic demand when dealing with ACL, a sensible strategy is to consider treatment options from the least to the most invasive ones. Bleaching has been considered the first line of treatment for discolored natural teeth since it is non-invasive, effective, predictable and cost-effective.[5] Moreover, dental bleaching does not exacerbate the mineral loss of existing caries lesions.[6,7] In this respect, we propose bleaching to be a suitable and advantageous option to aesthetically treat stained ACL. If successful, this approach may eliminate the need for more invasive aesthetic surgical treatments. In this report, we present the efficacy of dental bleaching systems to improve the color of stained ACL in extracted teeth (Part 1), as well as in a clinical case (Part 2).

Report

Part 1, Laboratory experiment

Ten previously extracted permanent human teeth with darkly-stained ACLs were chosen. They were stored in 0.1 % thymol (Thymol; Sigma-Aldrich Corporate, St Louis, USA) at 4 ºC in a refrigerator (Kenmore; Whirlpool, Michigan, USA) and obtained after local institutional review board approval (Indiana University IRB # NSO 911-07). ACLs were divided based on the surface of interest into pit and fissure (5 teeth) and smooth surface (5 teeth). Baseline images of the ACLs were captured by a digital camera (Nikon D3100; Nikon, Tokyo, Japan), under controlled light conditions and saved in JPG format. According to the manufacturer instructions, 15 % carbamide peroxide (Opalescence PF; Ultradent Products, Utah, USA) was applied (0.5 – 1.0 mm thick layer) on the stained surface and pit and fissures, and kept for 4 h per day. This procedure was performed for a period of six consecutive days (Figures 1.1 and 1.2). After each bleaching treatment, specimens were rinsed with running deionized water for 1 min and blotted-dried. During and after treatment, teeth were stored at 37 °C with an approximately 100 % relative humidity.

Part 2, Clinical case report

A 31-year-old medically fit female patient was seen at the Department of Cariology, Operative Dentistry and Dental Public Health, at Indiana University School of Dentistry.

The patient signed a written informed consent prior to screening and received oral soft and hard tissue examinations.

The patient reported dissatisfaction about her generalized yellowish teeth, along with the dark discoloration of ACLs present in the pits and fissures of her left maxillary and right mandibular 1st molars and 2nd premolars. In order to register the severity of the ACLs, they were classified using the International Caries Detection and Assessment System (ICDAS; grading details at: https://www.icdas.org).[8] Baseline scores ranged from 2 to 4 (Figure 1.3).

Focusing on the least invasive approach, treatment options for the pits and fissures were discussed, including bleaching or minimally invasive composite restorations. The patient indicated the desire to remove the discoloration, but declined any procedure that included tooth preparation. After explanation of potential risks and benefits of treatment options, the patient chose to perform in-office bleaching on her upper and lower yellowish stained teeth (anteriors and premolars), as well as on the discolored pits and fissures of the left maxillary and right mandibular 1st molars and 2nd premolars.

Teeth were cleaned with pumice (Pumice Preppies; Whip Mix Corporate, Kentucky, USA), air-dried and initial digital photographs were taken. 40 % hydrogen peroxide (Opalescence Boost; Ultradent Products, Utah, USA) was applied on the upper and lower teeth along with the discolored pits and fissures for 20 min, removed, and then applied again for another 20 min, and finally rinsed with water. After the second application, the

patient reported temporomandibular joint discomfort (due to the long chair-treatment time) along with mild sensitivity associated with the upper and lower incisors. No sensitivity was reported on the posterior teeth. The patient was satisfied with the overall whitening result and did not want to do a third cycle of bleaching. At the end of Parts 1 and 2, all teeth were photographed and re-evaluated. Figures 1.1, 1.2 and 1.3 show the visual improvement in color along with the ICDAS scores, for reference.

Discussion

Arrested carious lesions may present dark discoloration, but are otherwise inactive and require no surgical treatment unless they affect form, function and aesthetics.[9]

Discoloration can be due to the presence of trapped organic debris and metallic ions within the tooth structure, amino-acids released during proteolysis processes and food pigments uptake from the oral environment.[10] Studies have suggested that the blackish discoloration, in particular, results from the presence of ferric sulfide, a byproduct from the reaction between iron present in saliva or gingival fluid and hydrogen sulfide formed by bacteria.[11,12] To the authors' knowledge, the present report is the first attempt to aesthetically treat stained ACL by using dental bleaching systems. Part 1 (extracted teeth) employed a home bleaching agent based on a 15 % carbamide peroxide gel. There was a visual color improvement in most of the ACLs (Figures 1.1 and 1.2). The relatively low concentration of carbamide peroxide used required relatively long periods of contact with the tooth structure to be effective.

Based on these promising results of Part 1, and also on the wealthy of knowledge in the literature regarding the safety of dental bleaching procedures, this technique was applied in a selected clinical case. Higher concentration of bleaching agent (40 % hydrogen peroxide) was used, according to a standard in-office bleaching protocol.

This approach was considered primarily because the patient demanded fast bleaching results. In addition, it has the advantage of being site specific, prevents accidental gel ingestion, and avoids the discomfort associated with wearing trays.[13] After the second bleaching cycle (total of 40 min of application) the patient was satisfied with the overall bleaching results, and refused additional bleaching. It is possible that a third cycle specifically on the pits and fissures could potentially lead to further improvement of the color lightness.

The conservative approach showed notable visual color improvement in most of the ACLs in extracted teeth, and also clinically. Interestingly, this remarkable improvement in aesthetics also changed most of the baseline ICDAS scores, from one to three units toward a less extensive caries lesion code. It is noteworthy to mention that ICDAS is used for classification and assessment of caries progression, and not primarily intended for the assessment of color changes in the caries lesion, although lesion color is an important component of the visual examination. As an example, the ICDAS score for Sample S5 (Figure 1.1) was 4 at baseline and changed to 1 after bleaching. It is reasonable to assume that some dentists would surgically remove and restore the lesion at the baseline state.

Despite differences on treatments philosophies (whether to 'open' and restore or not), bleaching that particular lesion could directly affect the treatment decision, as not many dentists would surgically treat the same tooth after bleaching, based on color assessment. Cavitated lesions (ICDAS codes 3-6), on the other hand, may improve in color lightness after bleaching, yet their ICDAS scores will remain unchanged.

The results of this report show that the proposed procedure, although very useful from an aesthetic standpoint, may have potential implications on the use of ICDAS as a tool to monitor the progression/arrest of caries lesions. Further speculating, bleaching stained lesions may also negatively affect the ability of caries detection methods to distinguish sound from affected enamel (brown spot/ white spot lesions), especially those based on light excitation wavelengths differentiation such as fluorescence spectroscopy.[14]

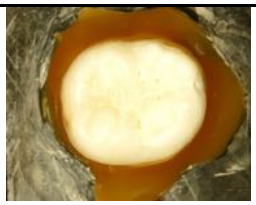
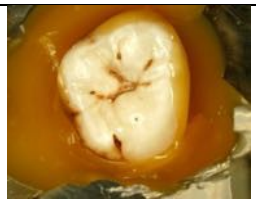
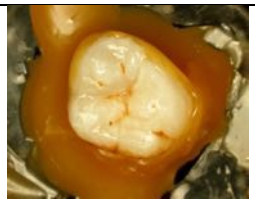
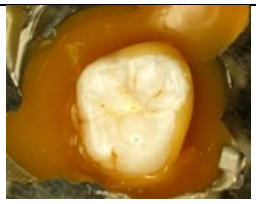
The color change during the bleaching treatment is related to the oxidative free radicals as they break the double bonds of the chromophore molecules,[15] alter their configurations and subsequently, change their optical properties.[15] This results in an improved color lightness that gradually blends with the surrounding tooth color. The color improvement seemed to be more noticeable in orange/brown discolorations compared to the dark brown/black ones. The different response of stains to bleaching agents, might be explained by the different stain history of each tooth (type, depth), therefore, the bleaching agent may not be as efficacious in different stained lesions.

Even if the final desired esthetic result is not achieved, the need for tissue removal would be less extensive resulting in a minimized restoration. In such case, immediate resin bonding to the bleached surface should be carefully considered, as it may reduce the restoration bonding strength[16] due to resin polymerization inhibition.[17,18]

Despite the aesthetic benefits, bleaching may also cause changes in the morphological structure of the inactive lesion, increasing its surface roughness[19] and possibly making it more prone for plaque accumulation. In such circumstances, there may be a potential risk for reactivation and progression of the caries lesion.[20] Therefore, implementation of preventive measures based on topical fluoride application may be necessary.[20] Other measures involving the use of sealants and resin infiltration, a technique that consists of filling/sealing the porosities present in the caries lesion,[21] can also be considered.[21] However, a similar concern on the potential polymerization inhibition, as mentioned above, also applies to these resin-based materials deserving further investigation.

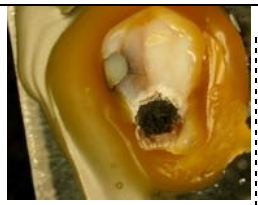
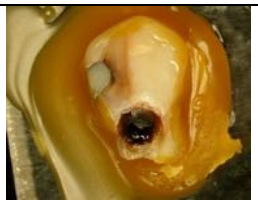
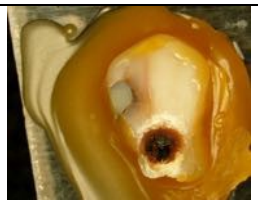
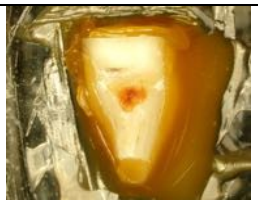
In conclusion, dental bleaching showed to be a promising conservative and cost effective aesthetic treatment for stained arrested caries lesions. Clinicians should carefully consider selecting appropriate cases based on safety and effectiveness.

Figure 1.1: Color improvement of arrested caries lesions (pit and fissure) treated with 15 % carbamide peroxide bleaching agent at baseline, day 2 and day 6.

Sample	Baseline	Day 2	Day 6
S1	 2*	 2*	 1*
S2	 2*	 1*	 0*
S3	 4*	 2*	 2*
S4	 2*	 2*	 1*
S5	 4*	 2*	 1*

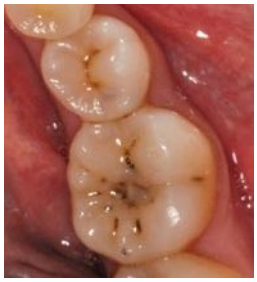
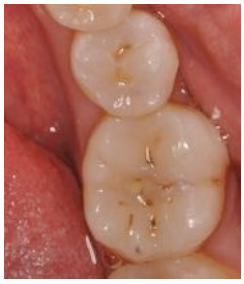
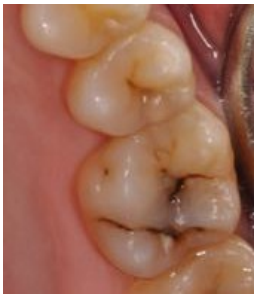
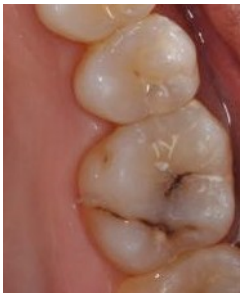
*ICDAS score: 0: Sound tooth; 1: First visual change in enamel; 2: Distinct visual change in enamel; 4: Underlying dentin shadow.

Figure 1.2: Color improvement of arrested caries lesions (smooth surface) treated with 15% carbamide peroxide bleaching agent at baseline, day 2 and day 6.

Sample	Baseline	Day 2	Day 6
S6	 6*	 6*	 6*
S7	 6*	 6*	 6*
S8	 6*	 6*	 6*
S9	 6*	 6*	 6*
S10	 6*	 6*	 6*

*ICDAS score: 6: Extensive cavity with visible dentin.

Figure 1.3: A case report showing color improvement of mandibular and maxillary arrested pits and fissures treated with 40 % hydrogen peroxide bleaching agent.

Sample	Baseline		After treatment	
2nd Premolar		4*		1*
1st Molar		4*		2*
2nd premolar		2*		1*
1st Molar		4*		4*

***ICDAS score: 1: First visual change in enamel; 2: Distinct visual change in enamel; 4: Underlying dentin shadow.**

References

1. Ahmad I. Risk management in clinical practice. Part 5. Ethical considerations for dental enhancement procedures. Br Dent J 2010;209:207-214.

2. Kelleher MG, Djemal S, Lewis N. Ethical marketing in'aesthetic' ('esthetic') or'cosmetic dentistry'. Part 1. Dent Update 2012;39:313-316, 318-320, 323-314 passim.

3. Schupbach P, Lutz F, Guggenheim B. Human root caries: histopathology of arrested lesions. Caries Res 1992;26:153-164.

4. Holmgren C, Gaucher C, Decerle N, Domejean S. Minimal intervention dentistry II: part 3. Management of non-cavitated (initial) occlusal caries lesions-non- invasive approaches through remineralisation and therapeutic sealants. Br Dent J 2014;216:237-243.

5. Sundfeld RH, Neto DS, Machado LS, de Oliveira FG, de Alexandre RS, Palo RM, Sundefeld ML. Dental bleaching with a 10% hydrogen peroxide product: A six-month clinical observation. Indian J Dent Res 2014;25:4-8.

6. Pinto CF, Paes Leme AF, Cavalli V, Giannini M. Effect of 10% carbamide peroxide bleaching on sound and artificial enamel carious lesions. Braz Dent J 2009;20:48-53.

7. Berger SB, Pavan S, Dos Santos PH, Giannini M, Bedran-Russo AK. Effect of bleaching on sound enamel and with early artificial caries lesions using confocal laser microscopy. Braz Dent J 2012;23:110-115.

8. Jallad M, Zero D, Eckert G, Ferreira Zandona A. In vitro Detection of Occlusal Caries on Permanent Teeth by a Visual, Light-Induced Fluorescence and

Photothermal Radiometry and Modulated Luminescence Methods. Caries Res 2015;49:523-530.

9. Chu CH, Lo EC. Microhardness of dentine in primary teeth after topical fluoride applications. J Dent 2008;36:387-391.

10. Watts A, Addy M. Tooth discolouration and staining: a review of the literature. Br Dent J 2001;190:309-316.

11. Reid JS, Beeley JA, MacDonald DG. Investigations into black extrinsic tooth stain. J Dent Res 1977;56:895-899.

12. Zyla T, Kawala B, Antoszewska-Smith J, Kawala M. Black stain and dental caries: a review of the literature. Biomed Res Int 2015;2015:469392.

13. Parinitha MS, Annapoorna BM, Sunil T, Suneeth S, Sowmya HK. Effect of power bleaching on the fluorosis stained anterior teeth case series. J Clin Diagn Res 2014;8:Zj01-03.

14. Buchalla W. Comparative fluorescence spectroscopy shows differences in noncavitated enamel lesions. Caries Res 2005;39:150-156.

15. Alqahtani MQ. Tooth-bleaching procedures and their controversial effects: A literature review. Saudi Dent J 2014;26:33-46.

16. Dishman MV, Covey DA, Baughan LW. The effects of peroxide bleaching on composite to enamel bond strength. Dent Mater 1994;10:33-36.

17. Josey AL, Meyers IA, Romaniuk K, Symons AL. The effect of a vital bleaching technique on enamel surface morphology and the bonding of composite resin to enamel. J Oral Rehabil 1996;23:244-250.

18. Moon HJ. Treatment after bleaching for optimal bonding. Restor Dent Endod 2015;40:177-178.

19. Melo CF, Manfroi FB, Spohr AM. Microhardness and roughness of enamel bleached with 10% carbamide peroxide and brushed with different toothpastes: an in situ study. J Int Oral Health 2014;6:18-24.

20. Arif N, Beighton D, Sheehy EC. Microbiological assessment of occlusal brown-spot lesions in primary molars. Caries Res 2006;40:398-402.

21. Kielbassa AM, Muller J, Gernhardt CR. Closing the gap between oral hygiene and minimally invasive dentistry: a review on the resin infiltration technique of incipient (proximal) enamel lesions. Quintessence Int 2009;40:663-681.

CHAPTER TWO

Aesthetic Management of Artificial Stained-Remineralized Enamel and Dentin Caries Lesions

Summary

Objective: Non-invasive aesthetic treatment options for stained arrested caries lesions have not been explored. This study aimed to develop laboratory models to create stained-remineralized caries-like lesions (s-RCLs) and to test the efficacy of bleaching on their aesthetic treatment.

Materials and Methods: Human dental specimens were embedded, and their color was measured spectrophotometrically at baseline and after demineralization. Specimens were randomly divided into four groups (n=14) based on the tested staining/remineralization protocols for a total of 5 days: G1-control (no staining/no remineralization); G2-no staining/remineralization in artificial saliva (AS); G3-non-metallic staining/AS; G4-metallic staining/AS. The mineral loss (ΔZ) and lesion depth (L) were measured using transverse microradiography along with color change (ΔE). Specimens were subjected to bleaching and color was re-evaluated. Data were analyzed using ANOVA models followed by Fisher's PLSD tests ($\alpha=0.05$).

Results: s-RCLs created in G4 were significantly ($p<0.001$) darker than G3, G2 and G1 regardless of substrate type and condition. s-RCLs in G2, G3 and G4 showed significantly lower ΔZ and L than G1 (all $p<0.001$), confirming their remineralized status.

G4 exhibited significantly lower ΔZ and L (better remineralization) compared to G2 ($p<0.001$). Bleaching treatment was more effective in non-metallic than metallic- stained lesions regardless of substrate type and condition ($p<0.001$).

Conclusion: The proposed models created distinct simulated s-RCLs. Non-metallic stained lesions were lighter and more responsive to bleaching compared to metallic stained lesions.

Clinical Relevance The developed experimental models will allow us to further investigate efficacy and safety of different aesthetic clinical management strategies of s-RCLs.

Introduction

Arrested caries lesions (ACLs) present highly mineralized surfaces [1], along with frequent and undesirable dark discolorations due to pigment incorporation in the remineralization process. The pigmentation may be exacerbated if remineralization is driven by a metallic salt, such as silver diamine fluoride [2]. Aesthetic management options for a stained ACL (s-ACL) include surgical interventions [3], such as dental microabrasion and placement of restorations [4]. However, microabrasion can cause significant loss of the tooth structure (up to 200 μm) [5], while inadequate restorations may result in poor marginal adaptation [6], plaque retention, sensitivity, secondary caries, and possibly periodontal disease [7].

We have previously proposed dental bleaching as a non-invasive option to aesthetically treat s-ACLs [8]. To further understand the clinical impact of this approach, it is necessary to test its efficacy. In vitro simulation allows for the creation of standardized stained lesions and for the use of very sensitive evaluation methods; both aspects that would not be possible to study under clinical conditions. Therefore, the development of an in vitro simulation model is necessary. Common discoloration causes of s-ACLs may involve food pigments, by- and end- products released during the proteolytic processes and the presence of trapped organic debris and metallic ions within the tooth structure [9]. Evidence shows that metallic stains are more difficult to treat [10, 11].

In the present study, we proposed two in vitro models to create stained-remineralized caries-like lesions (s-RCLs), based on the incorporation of metallic and non-metallic pigments during the remineralization of previously developed caries-like lesions [12-14]. These models will allow the study of efficacy and safety aspects related to the dental bleaching treatment of s-RCLs.

Materials and Methods

Experimental design

In this study we tested the simulation of two in vitro models for the creation of s-RCLs, on both enamel and dentin substrates. Artificial caries-like lesions were created, stained and then remineralized using different protocols, involving metallic and non-metallic staining. Sound enamel and dentin specimens were treated in parallel to understand differences between extrinsic (sound) and intrinsic (demineralized) staining and their aesthetic management. The following main factors were considered: staining/remineralization at four levels (negative control, positive control, non-metallic and metallic); and substrate condition at two levels (sound and demineralized). These factors were tested on both enamel and dentin substrates, independently.

The study outcomes were color change (ΔE) measured at three time points (after demineralization, staining/remineralization cycling, and bleaching); and mineral content (ΔZ) and lesion depth (L), measured after staining/remineralization cycling. Color

measurements were performed by spectrophotometry, while mineral loss and lesion depth were determined by transverse microradiography (TMR).

Specimen preparation

Enamel and root dentin slabs (4 × 4 × 2 mm^3) were sectioned from human molars (crown and root) using a low-speed diamond saw (Isomet, Buehler, Lake Bluff, IL, USA). Specimens were embedded in acrylic resin blocks (10×10×8 mm^3), (Varidur; High Performance Mounting Kit; Buehler, Lake Bluff, IL, USA), each block consisted of one slab of enamel and dentin (Figure A.1). They were flattened using a sequence of 500, 1200, 2400 and 4000 grit silicon carbide paper (MDFuga, Struers Inc., Cleveland, OH, USA), polished with 1-µm diamond suspension (Struers Inc., Cleveland, USA) then sonicated in a detergent solution (Micro-90, International Products Corporation, Burlington, USA).

Following the polishing procedure, the specimens were placed under running deionized water for 3 min. Specimens were stored at approximately 100% relative humidity at 4 ºC in a refrigerator (Kenmore; Whirlpool, MI, USA).

Caries-like lesion creation

Adhesive unplasticized polyvinyl chloride tapes (TapeCase Ltd., Wheeling, USA) were used to cover half of the surface (2 × 4 mm^2) of each specimen (enamel and dentin),

leaving the other half exposed. The lesion was initiated by placing specimens in a carboxymethylcellulose (CMC) demineralizing solution as described by Lippert et al., (2011) [15]; briefly, specimens were demineralized for seven days in a solution containing 0.1 M lactic acid, 4.1 mM Ca (as $CaCl_2 \cdot 2H_2O$), 8.0 mM PO_4 (as KH_2PO_4) and 1.0% w/v CMC (Sigma-Aldrich Co., St. Louis, MO, USA), with pH adjusted to 5.0, at 37°C.

Staining, remineralization and cycling

Artificial saliva solution (2.20 g/l gastric mucin, 1.45 mM $CaCl_2 \cdot 2H_2O$, 5.40 mM KH_2PO_4, 28.4 mM NaCl, 14.9 mM KCl, pH 7.0) was used as the remineralizing medium [16].

After demineralization, specimens were subdivided into four groups based on the different remineralizing protocols (Table 2.1). Group 1 (negative control) was stored in 100% relative humidity throughout the experiment, with no immersion in artificial saliva or any other treatment. Group 2 (positive control) was immersed in artificial saliva which was changed daily. Group 3 (non-metallic stain) was stored in a combined coffee (The Folger Coffee Company Inc., Orrville, OH, USA) and tea (Nestea, Nestle Inc., Glendale, CA, USA) solution prepared based on the manufacturer's instructions and used immediately after preparation. Specimens were kept in a container with a stirring bar in an incubator (~37°C) for 8 h of staining, then they were rinsed, allowed to air dry followed by application of 2% sodium fluoride (NaF) gel (Sultan

Healthcare Inc., York, PA, USA) for 4 min, before being immersed in artificial saliva overnight. Group 4 (metallic stain) was stored in a staining solution with iron based on the protocol described by Stookey et al. [17].

Specimens (Figure A.2) were placed on a rotating rod (~37°C incubator), which alternately exposed them to air and to a solution (800 ml / cycle, total of 4 l) consisting of trypticase soy broth, *Micrococcus luteus* BA13 (American Type Culture Collection, Manassas, VA, USA), coffee (Instant Folgers Crystals; Folgers, Orrville, OH, USA), tea (Nestea; Nestlé, Glendale, CA, USA), gastric porcine mucin (American Laboratories, Omaha, NE, USA), and ferric chloride (Fisher Scientific, Fair Lawn, NJ, USA). After each cycle (8 h/day) specimens were rinsed, allowed to air dry, followed by treatment with 38% SDF Advantage Arrest (Elevate Oral Care LLC, West Palm Beach, FL, USA) for 2 min [18] then immersed in artificial saliva overnight. During the 5 days of cycling, all specimens were stored in artificial saliva (replaced daily) overnight in an incubator.

Color assessment

L*a*b* (Commision Internationale de l'Eclairage) values were taken for each specimen. Measurements were performed using a spectrophotometer, Minolta Chroma meter CR-241 (Minolta Camera Co., Osaka, Japan) with a focus of 0.3 mm. Calibration of the spectrophotometer was conducted using a ceramic tile supplied by the manufacturer.

Color measurements were taken at baseline, and after demineralization, cycling and bleaching.

All measurements were repeated three times. The means of the L*a*b* values were measured and the color difference (ΔE) was calculated using the following equation, representing color changes after demineralization (ΔE_{Demin}: demineralization- baseline), cycling ($\Delta E_{Cycling}$: staining-demineralization) and bleaching ($\Delta E_{Bleaching}$: bleaching-staining): $\Delta E = \{(\Delta L^*)^2+(\Delta a^*)^2+(\Delta b^*)^2\}^{1/2}$, where ΔE is the color difference and ΔL*, Δa*, Δb* represent changes in lightness, red- green coordinate, and yellow-blue coordinate, respectively.

Lesion mineral content and lesion depth assessment

Specimens were mounted on acrylic rods and sectioned with a hard tissue microtome (Silverstone-Taylor Hard Tissue Microtome, Series 1000 Deluxe, USA). One section from enamel (100 μm +/- 20 μm) and dentin (180 μm +/- 20 μm) were obtained from each specimen, including both the sound and demineralized areas. The sections were kept moist to prevent dehydration, and were mounted with an aluminum step wedge on high- resolution glass plates Type I A (Microchrome Technology Inc., San Jose, Calif., USA) then X-rayed at 20 kV and 30 mA at a distance of 42 cm for 65 min.

The film was developed in a Kodak d-19 developer for 3 min, placed in a stop bath (Kodak 146-4247) for 45 s, and then fixed (Kodak 146-4106) for 3 min. All plates

were then rinsed in deionized water for 15 min and air dried. Microradiographs were examined with a Zeiss EOM microscope in conjunction with the TMR software v.3.0.0.11 (Inspektor Research Systems BV, Amsterdam, The Netherlands). A window (approx. 400 × 400 μm) representative of the entire lesion area and not containing any cracks, debris, or other alterations was selected for analysis. Sound enamel and sound dentin were defined at 87% [15] and 48% mineral volume respectively in order to determine mineral loss (ΔZ) and lesion depth (L).

Dental bleaching efficacy

After transverse microradiography (TMR) analysis, specimens (cut surface) were covered with acid-resistant nail varnish (Sally Hansen Advanced Hard as Nails Nail Polish, USA) except for the area exposed to the bleaching agent. Specimens were bleached using 40% hydrogen peroxide (pH 6.0 to 8.5) (Opalescence® Boost, Ultradent Products, Inc., South Jordan, UT, USA). A 0.5–1.0 mm thick layer of the bleaching gel was applied to the specimen surface, and kept for 20 min. Based on the manufacturer's instructions, the treatment consists of three 20 min applications for a total of 60 min of treatment time. After each application, the bleaching gel was wiped using a cotton pellet. Finally, after the third bleaching application each specimen was rinsed with running deionized water for 1 min, blotted dry then stored moist at 4°C (Figure A.3).

Statistical analysis

The color change calculated after demineralization, staining/remineralization cycling, and bleaching for each dental substrate was analyzed using three-way ANOVA, with factors for stain type, substrate condition and time points as well as all two-way and three-way interactions among the factors. Specimen and condition combination were allowed to have difference variances, and random effects were added to the model. The mineral loss and lesion depth change after treatment was analyzed using two-way ANOVA for each substrate, with factors for stain type and substrate condition as well as interaction between stain type and condition.

All pair-wise comparisons from ANOVA analysis were made using Fisher's Protected Least Significant Differences to control the overall significance level at 5%. Prior to the study, calculations showed that with a planned sample size of 14 per group, the study was designed to have 80% power to detect a 2.2 ΔE difference between groups, assuming a 5% significance level and standard deviation of 2.0.

Results

Color change

For both enamel and dentin, means of color change were significantly different among stain types, substrate conditions and time points (all $p<0.0001$). All two-way and three-way interactions among stain types, substrate conditions and time points were significant ($p<0.0001$), (ANOVA in appendix 1 and 2).

In lesioned enamel, G4 presented significantly ($p \leq 0.001$) darker stains after cycling compared to G3, G2 and G1, with no significant difference in control groups. G3 was more responsive ($p < 0.001$) to bleaching compared to G4, G1 and G2, however no differences were found in control groups (Table 2 . 2). In sound enamel, G4 presented significantly ($p \leq 0.001$) darker stains after cycling compared to G3, G2 and G1, however no significant difference was found in G3, G2 and G1. No significant differences were found among groups after bleaching (Table 2.3).

In lesioned and sound dentin, G4 showed significantly ($p \leq 0.001$) darker stains after cycling compared to G3, G2 and G1, with no significant difference in control groups. G3 was more responsive ($p < 0.001$) to bleaching compared to the other groups with no significant differences between them (Table 2.4 and 2.5).

The numerical values and comparisons within and among treatments for stain types, substrate conditions and time points for lesioned and sound enamel are found in Tables 2.2 and 2.3 respectively; while lesioned and sound dentin are in Tables 2.4 and 2.5 respectively.

Mineral loss and lesion depth

Significant differences were found for stain type, substrate condition and their

interaction (all $p<0.001$) (ANOVA in appendix 3, 4, 5 and 6), for both outcomes studied (mineral loss and lesion depth), in both enamel and dentin. Regarding lesioned enamel, G4 had the least significant ($p<0.001$) change in mineral loss and lesion depth (indicating SDF surface protective effect) compared to controls. G3 was not significantly different from G4 and G2, yet, significantly different ($p<0.001$) from G1 (Table 2.6).

In lesioned dentin, G4 had the least significant ($p<0.001$) change in mineral loss and lesion depth compared to G3, G2 and G1. Regarding mineral loss, groups: G3, G2 and G1 were significantly ($p<0.001$) different between each other. Regarding lesion depth, G3 and G2 were not significantly different yet significantly ($p<0.001$) different from G1 (Table 2.7).

The numerical values and comparisons within and among treatments for stain types and substrate conditions for enamel and dentin are in Tables 2.6 and 2.7 respectively.

Discussion

The different cycling protocols used in this study: artificial saliva (positive control), NaF (non- metallic stain) and SDF and iron (metallic stain) resulted in remineralized lesions with the stains (non-metallic, metallic) confined within the tooth structure, resulting in a model that may simulate a clinical case of s-RCL. In the present study, the color change after staining (ΔE range 5.3-49.3) was greater than 3.3 units. It is known that ΔE values

equal or greater than 3.3 are considered clinically perceptible, indicating that our staining process resulted in a distinct visual discoloration in all specimens [19].

Despite differences amongst study protocols, our TMR results were similar to other studies, as the three experimental groups (G2, G3 and G4) treated with artificial saliva, NaF and SDF respectively, showed significant remineralization compared to the negative control group G1 [12, 13, 15, 20, 21]. In the present study, we tested the remineralization efficacy by comparing treatment groups. We observed that SDF had lower ΔZ and L values compared to NaF which indicates that SDF is more effective than NaF in enhancing remineralization [22, 23].

This might be because the metallic model was remineralized using the higher fluoride concentration present in SDF compared to NaF (44,800 ppm F versus 905 ppm F). This is in agreement with previous studies, in which relatively high levels of fluoride were necessary to enhance remineralization and decrease demineralization [2, 23-27]. Furthermore, the mechanism of remineralization (SDF versus NaF) is different, as NaF facilitates remineralization by calcium, fluoride and phosphate deposition on the tooth surface to form calcium fluoride and fluoridated apatite crystals [28], which are acid resistant and present lower solubility than enamel [29, 30].

On the other hand, SDF forms a range of insoluble or sparingly soluble compounds including calcium fluoride, silver phosphate, and silver protein complexes [31, 32]. These precipitate on the dental surface, forming an insoluble protective layer that decreases

demineralization [31-34]. The overall combined effect of these factors might have played a substantial role in groups treated with SDF compared to NaF groups. This observation is in line with some reports suggesting that the synergistic effect of combining fluoride with other agents, including iron, calcium or tin, enhances remineralization [35, 36].

To better represent a clinical condition of a patient seeking professional bleaching, an in-office bleaching agent was used, as it is known to have high concentrations of hydrogen peroxide in order to penetrate deeper into the histological structure, increase the oxidative power and result in a fast bleaching result [37]. Metallic stains had substantial significant increase in ΔE (black discoloration) compared to non-metallic stains (orange/brown discoloration) but were less responsive to bleaching treatment compared to the non- metallic model, which had the highest significant improvement in color change compared to all groups.

The different response of non-metallic/metallic stains to the same bleaching treatment might be explained by the different stain type, depth and chemical composition of each model, which is in agreement with a previous study [8]. Therefore, the response to bleaching agent may not be as efficacious in G4 compared to G3. This can be explained by the bleaching process, as peroxide agents (highly unstable) dissociate upon tooth contact into water, oxygen, and free radicals. They increase the surface micro-porosities [38] resulting in enhanced oxidation of complex organic stains [37] represented in the orange/brown discoloration from coffee and tea in G3. However, the dark discoloration in G4 arose from the silver component in SDF and iron from the staining

solution, which cannot be broken down and bleached like organic chromophores [11]. This may support our findings of significantly better color improvement in non-metallic stains compared to metallic stains.

To better represent differences in staining susceptibility, we used different substrate conditions (sound versus demineralized). We observed that previously demineralized dental surfaces significantly incorporated more stains within the lesion during remineralization and were more difficult to bleach compared to sound substrates. This might be justified by the changes within the substrate structure throughout the demineralization process. As the pore size in the dental structure enlarged [39], it allowed for a substantial amount of non-metallic stains present in G3 and metallic stains in G4 during cycling to penetrate deeper and to be incorporated within the lesioned structure compared to the intact sound surface. These discolorations indicate that staining was successfully created within the tooth structure. This is in agreement with another study which found that during cycling, demineralized enamel surfaces were more susceptible to absorb external stains than sound enamel [40].

In general, dentin behaved similarly to enamel even though they were not directly comparable. However, the overall staining level in dentin was higher compared to enamel, especially in G4. Although both substrates were exposed to SDF, the difference in stain susceptibility might be explained by the substantial differences between their structures [41].

The darker stains in dentin might be due to the volume of silver in SDF and to the fact that SDF penetrated deeper into dentin structure, depositing more stain compared to enamel [11]. Caries lesions progress faster in dentin than in enamel, due to structural and compositional differences, which, in turn, results in more stain incorporation into dentin than enamel. Furthermore, the remineralization process in dentin is hampered because of the presence of organic matrix and therefore more difficult to achieve than in enamel [42]. As spontaneous mineral precipitation on the dentin organic matrix is unlikely to happen. [43]. Also, the structure of the collagen cannot be significantly denatured to serve as a scaffold for the growth of the mineral crystals [23]. Moreover, as the dentin carbonate ion substitutes for the phosphate ions in the crystal structure is high, it results in defects and calcium deficient regions, which negatively affects the remineralization process. This is supported by studies that have shown that approximately 1 out of 10 phosphate ions in enamel is replaced by carbonate compared to 1 out of 5 in dentin [44].

We proposed developing a stained-remineralized caries-like lesion model to facilitate testing bleaching treatment efficacy in vitro. Another application of this model includes using it as a screening tool. Despite the advantages of in vitro protocols with regards to their ability to be highly standardized and to allow for the control of different variables (lesion type, depth, stain), the significant limitation is their inability to simulate the complex biological processes involved in creating s-RCL.

In summary, the simulated s- RCLs proposed will allow us to further investigate mechanistic aspects related to the efficacy and safety of different bleaching treatments as conservative options for the aesthetic management of these lesions, in a quick, affordable and systematic fashion. It is essential to understand the different variables (stain types and remineralizing agents) that contribute to development of s-RCL models. The different stains (non- metallic and metallic) created light and dark discolorations respectively, in which the non-metallic model had better bleaching efficacy compared to the metallic model. These observations not only give us a better mechanistic understanding of the process, but also suggest specific clinical recommendations for the aesthetic management of stained-remineralized lesions.

Conclusion

The laboratory models developed in this investigation were able to create stained-remineralized caries like lesions in both enamel and dentin. Non-metallic stains were lighter in color and more responsive to bleaching treatment compared to metallic stains.

Table 2.1: Group definitions based on the staining and treatment protocol.

Groups	Type of staining	Remineralization protocol
G1 (Negative control)	No staining	No
G2 (Remin-control)	No staining	saliva
G3 (Non-Met)	Non-metallic stain	Saliva
G4 (Met)	Metallic stain	2% NaF

Table 2.2: Color change (ΔE) means (standard-deviation) after demineralization, cycling, and bleaching for lesioned enamel.

Groups	ΔE_{Demin} [+]		$\Delta E_{Cycling}$ [+]		$\Delta E_{Bleaching}$ [+]	
G1 (Negative control)	10.0 (1.9)	A/a	2.0 (1.1)	B/a*	5.2 (3.4)	C/a
G2 (Remin-control)	8.4 (3.0)	A/a	3.0 (3.9)	B/a*	4.4 (2.4)	B/a*
G3 (Non-Met)	8.8 (3.5)	A/a	21.9 (7.8)	B/b	24.1 (7.1)	B/b
G4 (Met)	13.0 (4.5)	A/b	49.3 (5.3)	B/c	14.6 (6.8)	A/c

Uppercase letters indicate significant difference within treatment (row, $p<0.05$); while lower case among treatments (column, $p<0.05$).

* Indicate no significant difference in enamel substrate condition (demineralized vs. sound).

[+] ΔE_{Demin}: demineralization-baseline, $\Delta E_{Cycling}$: staining-demineralization and $\Delta E_{Bleaching}$: bleaching-staining.

Table 2.3: Color change (ΔE) means (standard-deviation) after demineralization, cycling, and bleaching for sound enamel.

Groups	ΔE_{Demin} [+]		$\Delta E_{Cycling}$ [+]		$\Delta E_{Bleaching}$ [+]	
G1 (Negative control)	4.2 (2.4)	A/a	2.9 (1.1)	A/a*	10.2 (3.1)	B/a
G2 (Remin-control)	4.1 (2.6)	A/a	4.5 (3.0)	A/a*	7.2 (2.5)	B/a*
G3 (Non-Met)	4.9 (2.3)	A/a	5.3 (3.3)	A/a	8.9 (3.6)	B/a
G4 (Met)	3.3 (1.7)	A/a	24.6 (3.4)	B/b	8.3 (3.5)	C/a

Uppercase letters indicate significant difference within treatment (row, $p<0.05$); while lower case among treatments (column, $p<0.05$).

* Indicate no significant difference in enamel substrate condition (demineralized vs. sound).

[+] ΔE_{Demin}: demineralization-baseline, $\Delta E_{Cycling}$: staining-demineralization and $\Delta E_{Bleaching}$: bleaching-staining.

Table 2.4: Color change (ΔE) means (standard-deviation) after demineralization, cycling, and bleaching for lesioned dentin.

Groups	ΔE_{Demin} [+]		$\Delta E_{Cycling}$ [+]		$\Delta E_{Bleaching}$ [+]	
G1 (Negative control)	5.2 (3.2)	A/a	4.2 (2.4)	A/a*	11.6 (2.8)	B/a*
G2 (Remin-control)	3.7 (1.5)	A/b*	2.1 (1.3)	A/a*	10.6 (5.4)	B/a*
G3 (Non-Met)	3.9 (1.6)	A/ab	50.8 (9.8)	B/b	43.0 (9.8)	C/b
G4 (Met)	5.0 (2.4)	A/ab	58.0 (4.9)	B/c	11.4 (4.0)	C/a*

Uppercase letters indicate significant difference within treatment (row, $p<0.05$); while lower case among treatments (column, $p<0.05$).

* Indicate no significant difference in dentin substrate condition (demineralized vs. sound).

[+] ΔE_{Demin}: demineralization-baseline, $\Delta E_{Cycling}$: staining-demineralization and $\Delta E_{Bleaching}$: bleaching-staining.

Table 2.5: Color change (ΔE) means (standard-deviation) after demineralization, cycling, and bleaching for sound dentin.

Groups	ΔE_{Demin} [+]		$\Delta E_{Cycling}$ [+]		$\Delta E_{Bleaching}$ [+]	
G1 (Negative control)	2.3 (1.0)	A/a	2.3 (0.7)	A/a*	8.6 (3.2)	B/a*
G2 (Remin-control)	2.9 (2.3)	A/a*	3.1 (2.8)	A/a*	6.8 (2.4)	B/a*
G3 (Non-Met)	2.0 (1.0)	A/a	24.2 (6.6)	B/b	16.1 (5.3)	C/b
G4 (Met)	2.0 (1.5)	A/a	62.2 (3.7)	B/c	8.5 (3.0)	C/a*

Uppercase letters indicate significant difference within treatment (row, $p<0.05$); while lower case among treatments (column, $p<0.05$).

* Indicate no significant difference in dentin substrate condition (demineralized vs. sound).

[+] ΔE_{Demin}: demineralization-baseline, $\Delta E_{Cycling}$: staining-demineralization and $\Delta E_{Bleaching}$: bleaching-staining.

Table 2.6: TMR parameters (mineral loss, lesion depth) means (standard-deviation) of sound and lesioned enamel surface after different remineralizing treatments.

	ΔZ (vol%min × µm)				Lesion depth (µm)			
Groups	**Demin**		**Sound**		**Demin**		**Sound**	
G1 (Negative control)	970 (325)	A/a	288 (91)	B/a	53.6 (13.5)	A/a	17.3 (9.9)	B/a
G2 (Remin-control)	646 (114)	A/b	198 (62)	B/ab	36.6 (8.6)	A/b	10.5 (4.2)	B/b
G3 (Non-Met.)	550 (155)	A/bc	191 (73)	B/ab	31.1 (11.6)	A/bc	9.6 (3.4)	B/b
G4 (Met.)	468 (74)	A/c	178 (36)	B/b	25.8 (7.4)	A/c	8.9 (2.4)	B/b

Uppercase letters indicate significant difference between substrate condition (row, $p<0.05$), for each outcome measure. Lower case letters indicate significant difference among treatments (column, $p<0.05$).

Table 2.7: TMR parameters (mineral loss, lesion depth) means (standard-deviation) of sound and lesioned dentin surface after different remineralizing treatments.

	ΔZ (vol%min × μm)				Lesion depth (μm)			
Groups	**Demin**		**Sound**		**Demin**		**Sound**	
G1 (Negative control)	1595 (288)	A/a	389 (55)	B/a	102.9 (15.1)	A/a	21.5 (2.6)	B/a
G2 (Remin control)	1274 (166)	A/b	312 (50)	B/a	82.8 (14.2)	A/b	16.2 (1.9)	B/ab
G3 (Non-Met.)	1074 (163)	A/c	275 (92)	B/ab	76.3 (9.6)	A/b	14.4 (3.3)	B/ab
G4 (Met.)	819 (187)	A/d	194 (63)	B/b	61.4 (13.7)	A/c	9.1 (2.5)	B/b

Uppercase letters indicate significant difference between substrate condition (row, $p<0.05$), for each outcome measure. Lower case letters indicate significant difference among treatments (column, $p<0.05$).

References

1. Schupbach P, Lutz F and Guggenheim B (1992) Human root caries: histopathology of arrested lesions. Caries Research 26:153-64.

2. Rosenblatt A, Stamford TC and Niederman R (2009) Silver diamine fluoride: a caries "silver-fluoride bullet". Journal of Dental Research 88:116-25. doi: 10.1177/0022034508329406

3. Holmgren C, Gaucher C, Decerle N and Domejean S (2014) Minimal intervention dentistry II: part 3. Management of non-cavitated (initial) occlusal caries lesions-- non-invasive approaches through remineralisation and therapeutic sealants. British Dental Journal 216:237-43. doi: 10.1038/sj.bdj.2014.147

4. Mastroberardino S, Campus G, Strohmenger L, Villa A and Cagetti MG (2012) An Innovative Approach to Treat Incisors Hypomineralization (MIH): A Combined Use of Casein Phosphopeptide-Amorphous Calcium Phosphate and Hydrogen Peroxide-A Case Report. Case Reports in Dentistry 2012:379593. doi: 10.1155/2012/379593

5. Sundfeld RH, Sundfeld-Neto D, Machado LS, Franco LM, Fagundes TC and Briso AL (2014) Microabrasion in tooth enamel discoloration defects: three cases with long-term follow-ups. Journal of Applied Oral Science : revista FOB 22:347- 54.

6. Chandra PV, Harikumar V, Ramkiran D, Krishna MJ and Gouda MV (2013) Microleakage of class V resin composites using various self-etching adhesives: an in vitro study. The Journal of Contemporary Dental Practice 14:51-5.

7. Ababnaeh KT, Al-Omari M and Alawneh TN (2011) The effect of dental

restoration type and material on periodontal health. Oral Health & Preventive Dentistry 9:395-403.

8. Al-Angari SS and Hara AT (2016) A conservative approach to esthetically treat stained arrested caries lesions. Quintessence International (Berlin, Germany : 1985) 47:499-504. doi: 10.3290/j.qi.a36010

9. Watts A and Addy M (2001) Tooth discolouration and staining: a review of the literature. British Ddental Journal 190:309-16. doi: 10.1038/sj.bdj.4800959a

10. Iwamoto N, Shimada Y and Tagami J (2007) Penetration of silver nitrate into bleached enamel, dentin, and cementum. Quintessence International (Berlin, Germany : 1985) 38:e183-8.

11. Horst JA, Ellenikiotis H and Milgrom PL (2016) UCSF Protocol for Caries Arrest Using Silver Diamine Fluoride: Rationale, Indications and Consent. Journal of the California Dental Association 44:16-28.

12. Lee RC, Kang H, Darling CL and Fried D (2014) Automated assessment of the remineralization of artificial enamel lesions with polarization-sensitive optical coherence tomography. Biomedical Optics Express 5:2950-62. doi: 10.1364/boe.5.002950

13. Kielbassa AM, Shohadai SP and Schulte-Monting J (2001) Effect of saliva substitutes on mineral content of demineralized and sound dental enamel. Supportive care in cancer : Official Journal of the Multinational Association of Supportive Care in Cancer 9:40-7.

14. Lynch RJ, Mony U and ten Cate JM (2007) Effect of lesion characteristics and mineralizing solution type on enamel remineralization in vitro. Caries Research

41:257-62. doi: 10.1159/000101914

15. Lippert F, Lynch RJ, Eckert GJ, Kelly SA, Hara AT and Zero DT (2011) In situ fluoride response of caries lesions with different mineral distributions at baseline. Caries Research 45:47-55. doi: 10.1159/000323846

16. Al Dehailan L, Martinez-Mier EA and Lippert F (2015) The effect of fluoride varnishes on caries lesions: an in vitro investigation. Clinical Oral Investigations. doi: 10.1007/s00784-015-1648-4

17. Stookey GK, Burkhard TA and Schemehorn BR (1982) In vitro removal of stain with dentifrices. Journal of Dental Research 61:1236-9.

18. Yee R, Holmgren C, Mulder J, Lama D, Walker D and van Palenstein Helderman W (2009) Efficacy of silver diamine fluoride for Arresting Caries Treatment. Journal of Dental Research 88:644-7. doi: 10.1177/0022034509338671

19. Johnston WM and Kao EC (1989) Assessment of appearance match by visual observation and clinical colorimetry. Journal of Dental Research 68:819-22.

20. Preston KP, Smith PW and Higham SM (2008) The influence of varying fluoride concentrations on in vitro remineralisation of artificial dentinal lesions with differing lesion morphologies. Archives of Oral Biology 53:20-6. doi: 10.1016/j.archoralbio.2007.08.001

21. Hellen A, Mandelis A, Finer Y and Amaechi BT (2011) Quantitative evaluation of the kinetics of human enamel simulated caries using photothermal radiometry and modulated luminescence. Journal of Biomedical Optics 16:071406. doi: 10.1117/1.3564909

22. Mei ML, Li QL, Chu CH, Lo EC and Samaranayake LP (2013) Antibacterial

effects of silver diamine fluoride on multi-species cariogenic biofilm on caries. Annals of Clinical Microbiology and Antimicrobials 12:4. doi: 10.1186/1476- 0711-12-4

23. Mei ML, Ito L, Cao Y, Li QL, Lo EC and Chu CH (2013) Inhibitory effect of silver diamine fluoride on dentine demineralisation and collagen degradation. Journal of Dentistry 41:809-17. doi: 10.1016/j.jdent.2013.06.009

24. Karlinsey RL, Mackey AC, Dodge LE and Schwandt CS (2014) Noncontact remineralization of incipient lesions treated with a 5% sodium fluoride varnish in vitro. Journal of Dentistry for Children (Chicago, Ill) 81:7-13.

25. Gonzalez-Cabezas C, Jiang H, Fontana M and Eckert G (2012) Effect of low pH on surface rehardening efficacy of high concentration fluoride treatments on non- cavitated lesions. Journal of Dentistry 40:522-6. doi: 10.1016/j.jdent.2012.03.002

26. Nantanee R, Santiwong B, Trairatvorakul C, Hamba H and Tagami J (2016) Silver diamine fluoride and glass ionomer differentially remineralize early caries lesions, in situ. Clinical Oral Investigations 20:1151-7. doi: 10.1007/s00784-015- 1603-4

27. Zhi QH, Lo EC and Kwok AC (2013) An in vitro study of silver and fluoride ions on remineralization of demineralized enamel and dentine. Australian Dental Journal 58:50-6. doi: 10.1111/adj.12033

28. Cury JA and Tenuta LM (2008) How to maintain a cariostatic fluoride concentration in the oral environment. Advances in Dental Research 20:13-6.

29. Tenuta LM and Cury JA (2010) Fluoride: its role in dentistry. Brazilian Oral Research 24 Suppl 1:9-17.

30. Lata S, Varghese NO and Varughese JM (2010) Remineralization potential of fluoride and amorphous calcium phosphate-casein phospho peptide on enamel lesions: An in vitro comparative evaluation. Journal of Conservative Dentistry : JCD 13:42-6. doi: 10.4103/0972-0707.62634

31. Yu DG, Kimura Y, Fujita A, Hossain M, Kinoshita JI, Suzuki N and Matsumoto K (2001) Study on acid resistance of human dental enamel and dentin irradiated by semiconductor laser with Ag(NH3)2F solution. Journal of Clinical Laser Medicine & Surgery 19:141-6. doi: 10.1089/10445470152927973

32. Knight GM, McIntyre JM, Craig GG, Mulyani, Zilm PS and Gully NJ (2009) Inability to form a biofilm of Streptococcus mutans on silver fluoride- and potassium iodide-treated demineralized dentin. Quintessence International (Berlin, Germany : 1985) 40:155-61.

33. Liu BY, Lo EC and Li CM (2012) Effect of silver and fluoride ions on enamel demineralization: a quantitative study using micro-computed tomography. Australian Dental Journal 57:65-70. doi: 10.1111/j.1834-7819.2011.01641.x

34. ten Cate JM, Buijs MJ, Miller CC and Exterkate RA (2008) Elevated fluoride products enhance remineralization of advanced enamel lesions. Journal of Dental Research 87:943-7.

35. Zhang Y and Huang RZ (2016) [Study on enhancement effect and mechanism of the acid resistance of enamel by Fe2+ and F]. Shanghai kou qiang yi xue. Shanghai Journal of Stomatology 25:42-6.

36. Byeon SM, Lee MH and Bae TS (2016) The effect of different fluoride application methods on the remineralization of initial carious lesions. Restorative

Dentistry & Endodontics 41:121-9. doi: 10.5395/rde.2016.41.2.121

37. Bortolatto JF, Pretel H, Floros MC, Luizzi AC, Dantas AA, Fernandez E, Moncada G and de Oliveira OB, Jr. (2014) Low Concentration H(2)O(2)/TiO_N in Office Bleaching: A Randomized Clinical Trial. Journal of Dental Research 93:66s-71s. doi: 10.1177/0022034514537466

38. El-Murr J, Ruel D and St-Georges AJ (2011) Effects of external bleaching on restorative materials: a review. Journal (Canadian Dental Association) 77:b59.

39. Kidd EA and Fejerskov O (2004) What constitutes dental caries? Histopathology of carious enamel and dentin related to the action of cariogenic biofilms. Journal of Dental Research 83 Spec No C:C35-8.

40. Ahrari F, Akbari M, Mohammadpour S and Forghani M (2015) The efficacy of laser-assisted in-office bleaching and home bleaching on sound and demineralized enamel. Laser Therapy 24:257-64. doi: 10.5978/islsm.15-OR-15

41. Cadavid AS, Lince CM and Jaramillo MC (2010) Dental caries in the primary dentition of a Colombian population according to the ICDAS criteria. Brazilian Oral Research 24:211-6.

42. Chen Z, Cao S, Wang H, Li Y, Kishen A, Deng X, Yang X, Wang Y, Cong C, Wang H and Zhang X (2015) Biomimetic remineralization of demineralized dentine using scaffold of CMC/ACP nanocomplexes in an in vitro tooth model of deep caries. PloS One 10:e0116553. doi: 10.1371/journal.pone.0116553

43. Klont B and ten Cate JM (1991) Susceptibility of the collagenous matrix from bovine incisor roots to proteolysis after in vitro lesion formation. Caries Research 25:46-50.

44. Featherstone JD (2008) Dental caries: a dynamic disease process. Australian Dental Journal 53:286-91. doi: 10.1111/j.1834-7819.2008.00064.x

CHAPTER THREE

Dental Bleaching Efficacy and Impact on Demineralization Susceptibility of Artificially-Induced Stained-Remineralized Caries Lesions

Summary

Objectives: To evaluate the efficacy of different bleaching systems on artifically created stained-remineralized caries lesions; and to assess the susceptibility of bleached lesions to further demineralization.

Material and Methods: Human enamel specimens were sectioned, polished, demineralized, and randomly divided into 6 groups (n= 21) to create either non-metallic (non-Met: G1, G2 and G3) or metallic (Met: G4, G5 and G6) stained/remineralized lesions. G1 and G4 received no treatment, while G2 and G5 were treated with 15% carbamide peroxide (at-home bleaching protocol; 4h/day x 7), and G3 and G6 with 40% hydrogen peroxide (in-office bleaching protocol; 20min x 3). Lesion mineral loss and depth were measured by transversal microradiography, color change by spectrophotometry. Susceptibility to demineralization was tested after bleaching treatment. Outcomes were analyzed using ANOVA models followed by Fisher's PLSD tests (α=0.05).

Results: Metallic-stained lesions were significantly darker (all $p<0.001$) and more difficult to bleach ($p<0.005$) than non-Met ones. For both stain types, at-home bleaching protocol was more effective than in-office ($p<0.005$); however, it also increased the lesion susceptibility to demineralization ($p<0.05$). After bleaching, non-Met lesions were more susceptible to demineralization compared to Met lesions ($p<0.05$).

Conclusion: At-home bleaching protocol (15% CP) presented greater bleaching efficacy compared to in-office bleaching protocol (40% HP). However, it was associated with increased susceptibility of enamel to subsequent demineralization especially in non-Met lesions.

Clinical Relevance: Bleaching stained-arrested caries lesions may lead to aesthetically satisfactory results depending on the type of stain involved, as well as on the bleaching system used.

Introduction

Aesthetic dentistry is considered an essential part of the restorative dental practice [1], yet options regarding aesthetic treatment of stained arrested caries lesions are few and mostly confined to surgical intervention [2]. The major drawback of invasive approaches is the potential contribution to the repeat restoration cycle [3]. Therefore, the development of minimally invasive treatment options would benefit the aesthetic treatment of stained arrested caries lesions.

Dental bleaching is considered a non-invasive, effective, safe, predictable and inexpensive procedure that may be indicated for the aesthetic treatment of these discolorations [4]. Despite the benefits of color improvement, bleaching has been reported to negatively affect the physical properties of the tooth structure, by decreasing surface hardness and increasing surface porosity [5, 6]. These changes have shown to potentially increase the susceptibility of the bleached enamel substrate to further demineralization [7].

We have previously reported dental bleaching to successfully improve the aesthetics of naturally stained arrested caries lesions [8]. We further explored this concept by developing a stained-remineralized-caries-like lesion (s-RCL) model and characterizing some of the enamel physical properties. In this study, we evaluated the efficacy of different bleaching systems on the artificially created s-RCLs utilizing different stain types, and we also assessed their susceptibility to further demineralization.

Materials and Methods

Experimental design

This study investigated three factors: stain type at two levels (non-metallic and metallic), bleaching treatment at three levels (control, at-home and in-office), and the susceptibility of bleached s-RCL to demineralization at two levels (yes and no). The experimental units were human enamel slabs embedded in acrylic blocks and polished (n= 21). The study outcomes were color change (ΔE) measured at 3 time points (after demineralization, cycling and bleaching); mineral content (ΔZ) and lesion depth (L) measured after cycling; and mineral content change (ΔΔZ) and lesion depth change (ΔL) measured after a second demineralization. Color measurements were performed by spectrophotometry, while mineral loss and lesion depth were measured by transverse microradiography (TMR).

Specimen preparation

Enamel slabs ($4 \times 4 \times 2$ mm^3) were sectioned from human molars using a low-speed diamond saw (Isomet, Buehler, Lake Bluff, IL, USA). After collection and during the preparation process, the teeth were stored in 0.1% thymol solution. The bottom and top sides of the slabs were sequentially ground flat using silicon carbide grinding papers (Struers RotoPol 31/RotoForce 4 polishing unit, USA). One hundred twenty six specimens were embedded in an acrylic resin (Varidur; High Performance Mounting Kit;

Buehler, Lake Bluff, IL, USA). The specimen surface was flattened and sequentially ground using #500-, 1200-, 2400- and 4000-grit silicon carbide papers (MDFuga, Struers Inc., Cleveland, OH, USA), polished with 1-μm diamond suspension (Struers Inc., Cleveland, USA), then sonicated in a detergent solution (Micro-90, International Products Corporation, Burlington, USA). Following the polishing procedure, the specimens were placed under running deionized water for 3 min. Specimens were stored at approximately 100% relative humidity at 4 ºC.

Caries-like lesion creation

Enamel caries-like lesions were created using the protocol described by Lippert et al., (2011) [9]. Briefly, specimens were immersed for seven days in a solution containing 0.1 M lactic acid, 4.1 mM Ca (as $CaCl_2 . 2H_2O$), 8.0 mM PO_4 (as KH_2PO_4) and 1.0% w/v carboxymethylcellulose (Sigma-Aldrich Co., St. Louis, MO, USA), pH adjusted to 5.0 using KOH at 37°C. Specimens were rinsed, dried, then stored at 100% relative humidity at 4 °C until use.

Staining, remineralization and cycling

After demineralization, specimens were subdivided into six groups (Table 3.1). Groups 1, 2 and 3 (non-metallic stain) were stored in a staining solution containing coffee (The Folger Coffee Company Inc., Orrville, OH, USA) and tea (Nestle Inc., Glendale, CA, USA) freshly prepared. Specimens were kept in a container with a stirring bar in an

incubator (~37°C) for the 8 h of staining, then they were rinsed, allowed to air dry followed by application of 2% sodium fluoride (NaF) gel (Sultan Healthcare Inc., York, PA, USA) for 4 min, before being immersed in saliva over night. Groups 4, 5 and 6 (metallic stain) were stained based on the protocol described by Stookey et al. [10]

Specimens were placed on a rotating rod (~37°C incubator), which alternately exposed them to air and to a solution consisting of trypticase soy broth, Micrococcus luteus BA13 (American Type Culture Collection, Manassas, VA, USA), coffee (Instant Folgers Crystals; Folgers, Orrville, OH, USA), tea (Nestea; Nestlé, Glendale, CA, USA), gastric porcine mucin (American Laboratories, Omaha, NE, USA), and ferric chloride (Fisher Scientific, Fair Lawn, NJ, USA). After each cycle (8 h/day) specimens were rinsed, allowed to air dry, followed by treatment with 38% silver diamine fluoride (SDF; Advantage Arrest, Elevate Oral Care LLC, West Palm Beach, FL, USA) for 2 min [11] then immersed in saliva over night.

Artificial saliva solution (2.20 g/l gastric mucin, 1.45 mM $CaCl_2.2H_2O$, 5.40 mM KH_2PO_4, 28.4 mM NaCl, 14.9 mM KCl, pH 7.0) [9] was used as the remineralizing medium. During the 5 days of cycling, all groups (1 to 6) were stored in artificial saliva (replaced daily) overnight in an incubator.

Color assessment

L*a*b* values (Commision Internationale de l'Eclairage) were taken for each specimen at baseline, and after demineralization, cycling and bleaching. All measurements were repeated 3 times, and performed using a spectrophotometer, Minolta Chroma meter CR-241 (Minolta Camera Co., Osaka, Japan), with a light beam diameter of 0.3 mm. Calibration of the spectrophotometer was conducted using a ceramic tile supplied by the manufacturer.

The means of the L*a*b* values were measured and the color difference (ΔE) was calculated using the following equation, representing color changes after demineralization (ΔE_{Demin}: demineralization-baseline), cycling ($\Delta E_{Cycling}$: staining-demineralization) and bleaching ($\Delta E_{Bleaching}$: bleaching-staining):

$$\Delta E = \{(\Delta L^*)^2+(\Delta a^*)^2+(\Delta b^*)^2\}^{1/2}$$

where ΔE is the color difference and ΔL*, Δa*, Δb* represent changes in lightness, red-green coordinate, and yellow-blue coordinate, respectively.

Lesion mineral content and lesion depth assessment

Specimens were mounted on plastic rods and sectioned with a hard tissue microtome (Silverstone-Taylor Hard Tissue Microtome, Series 1000 Deluxe, USA). One section, 100 μm +/- 20 μm, was obtained from each specimen. The sections were kept moist to prevent dehydration, and were mounted with an aluminum step wedge on high-resolution glass plates Type I A (Microchrome Technology Inc., San Jose, Calif., USA) then X-rayed at 20 kV and 30 mA at a distance of 42 cm for 65 min.

The exposed plate was developed (in a Kodak d-19 developer) for 3 min, placed in a stop bath (Kodak 146-4247) for 45 s, and then fixed (Kodak 146-4106) for 3 min. All plates were then rinsed in deionized water for 15 min and air-dried. Microradiographs were examined under a microscope (Zeiss EOM, Hitachi Denshi Ltd., Thornwood, NY, USA) in conjunction with the TMR software v.3.0.0.11 (Inspektor Research Systems BV, Amsterdam, The Netherlands). A region of interest (approx. 400 × 400 μm) representative of the entire lesion area and not containing any cracks, debris, or other artifacts was selected for analysis. Sound enamel was defined at 87% mineral volume [12] in order to determine overall mineral loss (ΔZ) and mean lesion depth (L).

Dental bleaching efficacy test

Group 1 and 4 (control) were kept moist in an incubator for 8 h, then stored moist at 4°C. Group 2 and 5 were treated with at- home bleaching protocol, 15% carbamide peroxide (CP) home bleaching agent (pH 6.5), (Opalescence PF, Ultradent Products, Inc., South Jordan, UT, USA). The bleaching gel was painted on the top surface of each specimen (0.5–1.0 mm thick) and kept for 4 h each day [1] in an incubator at 37°C for a total treatment of 1 week.

After bleaching, specimens were rinsed with running distilled water for 1 min to remove the bleaching agents, blot dried and stored in a moist environment at 4°C. Group 3 and 6 were bleached using in-office bleaching protocol, using 40% hydrogen peroxide (pH 6.0 to 8.5) (Opalescence® Boost, Ultradent Products, South Jordan, UT, USA). A 0.5–1.0 mm

thick layer of the bleaching gel was applied to the specimen surface, allowing the gel to remain on the teeth for 20 min. Based on the manufacturers instructions, the treatment consisted of three 20 min applications for a total of 60 min of treatment time

After bleaching, specimens were rinsed with running distilled water for 1 min to remove the bleaching agents, blot dried and stored in a moist environment at 4°C. Group 3 and 6 were bleached using in-office bleaching protocol, using 40% hydrogen peroxide (pH 6.0 to 8.5) (Opalescence® Boost, Ultradent Products, South Jordan, UT, USA). A 0.5–1.0 mm thick layer of the bleaching gel was applied to the specimen surface, allowing the gel to remain on the teeth for 20 min. Based on the manufacturers instructions, the treatment consisted of three 20 min applications for a total of 60 min of treatment time.

After each cycle, the bleaching gel was wiped by a cotton pellet. Finally, after the third bleaching cycle, each specimen was rinsed with running deionized water for 1 min. blot dried then stored moist at 4°C.

Susceptibility to demineralization test

Each group (n=21) was further divided into two subgroups, either submitted to second demineralization (n=11) or not (n=10).

The sides of each sample (demineralized group) was covered by nail polish, and further demineralized utilizing the same demineralization solution used previously (CMC) for a

total of 7 days. TMR verified the final mineral content of the lesions, and the mineral loss change ($\Delta\Delta Z$) and lesion depth change (ΔL) were calculated.

Statistical analysis

The color changes (after demineralization, after cycling, and after bleaching), mineral loss and lesion depth change (after cycling and after 2nd demineralization) were analyzed using four-way ANOVA, with factors for stain type, bleach treatment, susceptibility to 2nd demineralization, and time points, as well as all two-way, three-way and four-way interactions among the factors. Same specimen measured at different time was allowed to have difference variances.

The mineral loss change ($\Delta\Delta Z$) and lesion depth change (ΔL) were analyzed using three-way ANOVA with factors for stain type, bleach treatment and susceptibility to 2nd demineralization, as well as two-way and three-way interactions among the factors. All pair-wise comparisons from ANOVA analysis were made using Fisher's Protected Least Significant Differences to control the overall significance level at 5%.

Results

Color change

Means of color change (ΔE) were significantly different within stain types, bleaching treatments and time points ($p<0.0001$), but was not within susceptibility to 2nd demineralization (ANOVA in appendix 7).

Metallic stains (G1, G2 and G3) presented significantly ($p\leq0.001$) darker stains after staining/remineralization cycling compared to non-metallic stains, however they were significantly more difficult to bleach ($p\leq0.005$). At-home bleaching protocol in both non-metallic and metallic groups (G2 and G5) was more ($p<0.005$) effective (indicating lighter color) than in-office bleaching protocol. The mean values of ΔE and comparisons within and among treatments for stain types, bleaching treatments and time points are in Table 3.2.

Mineral loss and lesion depth

The mineral loss (ΔZ) after the staining/remineralization/cycling, and mineral loss change (ΔΔZ) after 2nd demineralization were significantly different regarding stain types, bleaching treatments, susceptibility to 2nd demineralization and time points ($p\leq0.05$). (ANOVA in appendix 8 and 9). The lesion depth (L) and lesion depth change (ΔL) after cycling and 2nd demineralization was significantly different regarding stain types, bleaching treatments, susceptibility to 2nd demineralization and time points ($p\leq0.001$). (ANOVA in appendix 10 and 11).

After the 2nd demineralization, the non-demineralized bleached subgroups: G2, G3, G5 and G6 (non-metallic/metallic) had significantly ($p \leq 0.05$) more mineral loss change ($\Delta\Delta Z$) compared to G1 and G4 (control). After cycling, ΔZ and L were significantly ($p \leq 0.05$) lower in metallic stains compared to non-metallic stains for all groups, which indicate a better remineralization of SDF compared to NaF.

Within each treatment subgroup (G1-G6), demineralized subgroups had significant increase in $\Delta\Delta Z$ ($p<0.001$) and ΔL ($p \leq 0.05$) compared to non-demineralized subgroups (more susceptible to demineralization) except for $\Delta\Delta Z$ in G6 and ΔL in G5. Regarding subgroups submitted to 2nd demineralization, non-metallic stains (G1,G2 and G3) had significant increase in $\Delta\Delta Z$ compared to metallic stains (G4,G5 and G63) in regards to in-office and at-home bleaching groups ($p<0.05$). Within bleaching treatments submitted 2nd demineralization, at-home bleaching protocol (metallic/non-metallic groups) demonstrated significant increase in $\Delta\Delta Z$ ($p<0.001$) compared to in-office $\Delta\Delta Z$ ($p=0.05$). The numerical values and comparisons within and among treatments for stain types, bleaching treatments and time points are in Table 3.3 and 3.4 respectively.

Discussion

This study tested the efficacy and safety of two different bleaching systems using a previously developed remineralized-stained caries like-lesions in-vitro model. This allowed standardization of the lesions reducing variability, and a systematic and well controlled test of color change, mineral loss and lesion depth.

Color change (ΔE) was used to verify the creation of stained lesions and bleaching efficacy of the tested systems/techniques. Johnston and Kao (1989) speculated that ΔE values exceeding 3.3 units are clinically perceptible [13]. In this regard, the ΔE range in this study after staining (non metallic: 16.7-18.5; metallic: 58.2-59.1) suggests remarkable clinically visible changes. Both the Non-Met and Met conditions had a significant increase in ΔE becoming darker, as indicated by the decrease in L* values (Appendix 12) compared to the color measured before the staining/remineralizing cycles. These results indicated successful incorporation of the stains during remineralization of the created lesions.

Metallic stained lesions were significantly darker than non-metallic, which can be explained by the different chemical composition (organic versus metallic stains) and depth of the created lesions [8]. They were also significantly more difficult to bleach, possibly due to the mechanism of action of peroxides, as they tend to oxidize organic chromogens present in non-metallic stains (orange/brown). Conversely, metallic compounds can not be easily degraded by oxidation [8, 14].

The bleaching products tested contained 15% CP (at-home) or 40% HP (in-office). At-home bleaching protocol caused significant color improvement in both metallic and non-metallic s-RCLs compared to in-office, despite the difference in concentration of hydrogen peroxide (15% CP (~5% HP) versus 40% HP). However, it should be kept in mind that the at home bleaching protocol had longer time exposure (4 versus 1 h) and application duration (7 versus 1 day), which resulted in a total of 28 h exposure compared

to only 1 h of the in-office treatment. This agrees with previous studies suggesting that longer enamel bleaching time caused better color improvement regardless of the concentration of bleaching agents [15-20]. In this regard, in-office bleaching systems with longer application time might be more appropriate for this type of application and should be considered in future tests.

Existing literature reports mixed findings considering the side effects of bleaching on the increase of enamel susceptibility to demineralization [5, 7, 14, 21]. The contrasting results may be due to study variables, such as substrate type, the concentration, contact and treatment time of the bleaching gels and different evaluation techniques, among other factors. In our study, we observed that the mineral content change ($\Delta\Delta Z$) of subgroups not subjected to 2nd demineralization was significantly higher in bleached groups compared to control in both groups (non-metallic and metallic). As the bleaching agent contacts the tooth structure it develops focal areas of very shallow demineralization [22]. This indicated that bleaching promoted mineral loss from enamel structure, which is in agreement with previous studies [6, 23, 24].

However, studies have shown that the increase in enamel susceptibility to demineralization after bleaching can be reduced when specimens were stored in saliva (human or artificial), simulating what would happen in the mouth [25-27]. Saliva was not used during bleaching in our study, as we focused on the effect of different bleaching systems solely by eliminating other variables such as saliva and fluoride. Further studies

could be established by introducing saliva during bleaching to evaluate its effect on remineralization.

After the 2nd demineralization, non-metallic stained lesions showed significantly more mineral loss (ΔΔZ) when bleached by the at-home protocol (G2) followed by in-office (G3) and then the control group, which supports our findings in bleaching. At-home bleaching protocol had longer surface time exposure, which resulted in deeper penetration of the gel into the tooth structure and oxidizing more stains. This process resulted in better color improvement, but also undesirably higher mineral loss change compared to in-office [28]. Metallic groups also represented significantly more mineral loss and lesion depth when treated with at-home bleaching protocol, which is consistent with the findings of the non-metallic groups. This might be explained by the SDF insoluble surface protective layer, which caused a lower response to the bleaching agent, and minimized demineralization within the lesions [29].

In an attempt to combine efficacy and safety, dental practitioners should carefully consider selecting appropriate cases of s-RCLs treated with bleaching agents, as darker stains treated with in-office bleaching protocol might need further bleaching treatment (more than one visit) or at home bleaching for longer time periods to produce clinically satisfactory esthetic results which may replace the traditional surgical treatment.

Conclusion

Within the limitations of this in vitro study, we concluded that remineralized-stained lesions treated with at-home bleaching protocol (15% CP) resulted in greater efficacy in color improvement compared to an in-office bleaching protocol (40% HP). However, it was associated with increased susceptibility to subsequent enamel demineralization, especially in non-met stained lesions.

Table 3.1: Group definitions based on the staining, treatment and bleaching protocol.

Groups	Type of staining	Remineralization treatment	Bleaching protocol	2nd demineralization
G1	Non-Metallic	2% NaF	Control	Yes
				No
G2	Non-Metallic	2% NaF	At-home	Yes
				No
G3	Non-Metallic	2% NaF	In-Office	Yes
				No
G4	Metallic	38% SDF	Control	Yes
				No
G5	Metallic	38% SDF	At-home	Yes
				No
G6	Metallic	38% SDF	In-Office	Yes
				No

2% NaF: Sodium fluoride gel, SDF: Silver diamine fluoride solution, At-home: 15% Carbamide peroxide bleaching gel, In-Office: 40% Hydrogen peroxide bleaching gel.

Table 3.2: Color change (ΔE) means (standard-deviation) after demineralization, staining, and bleaching.

		Groups	ΔE Demin[+]		ΔE Staining[+]		ΔE Bleaching[+]	
Remineralizing/Staining protocol	Non-Metallic	G1 (Control)	4.0 (2.0)	A/ab	18.5 (3.1)	B/a	5.6 (4.0)	A/a*
		G2 (At-home)	3.0 (1.5)	A/a	16.7 (3.8)	B/a	20.6 (3.9)	C/b
		G3 (In-office)	5.0 (2.3)	A/b*	17.3 (3.6)	B/a	17.1 (3.5)	B/c
	Metallic	G4 (Control)	6.4 (3.8)	A/a	58.3 (4.8)	B/a	8.3 (4.2)	A/a*
		G5 (At-home)	5.0 (3.0)	A/a	58.2 (3.2)	B/a	29.0 (4.2)	C/b
		G6 (In-office)	6.2 (2.9)	A/a*	59.1 (3.9)	B/a	23.3 (6.6)	C/c

Uppercase letters indicate significant difference within treatment (row, $p<0.05$); while lower case between treatments (column, $p<0.05$).

*: Indicate no significant difference between stain type (Non-metallic vs. Metallic).

[+] ΔE_{Demin}: demineralization-baseline, $\Delta E_{Staining}$: staining-demineralization and $\Delta E_{Bleaching}$:

bleaching-staining.

Table 3.3: Lesion mineral content (ΔZ) means (standard-deviation) after remineralization/staining cycling, and mineral content change (ΔΔZ) after second demineralization.

ΔZ (vol%min× µm)					ΔΔZ (vol%min × µm)			
Groups			After cycling		Non-demin		Demin	
Remineralizing/Staining protocol	Non-Metallic	G1 (Control)	525 (87)	§	-11 (12)	A/a*	121 (72)	B/a*
		G2 (At-home)	564 (212)	§	96 (91)	A/b*	313 (188)	B/b
		G3 (In-office)	557 (75)	§	75 (43)	A/b*	206 (98)	B/c
	Metallic	G4 (Control)	460 (47)	§	-8 (42)	A/a*	126 (43)	B/a*
		G5 (At-home)	474 (49)	§	93 (33)	A/b*	205 (73)	B/b
		G6 (In-office)	487 (62)	§	70 (34)	A/b*	132 (45)	A/a

Uppercase letters indicate significant difference within treatment (row, $p<0.05$); while lower case among treatments (column, $p<0.05$).

*: Indicate no significant difference between stain type (Non-metallic vs. Metallic).

§: Indicate significant difference between stain type.

[+]: $\Delta\Delta Z$ = ΔZ TMR bleaching/2nd demin – ΔZ TMR cycling.

Table 3.4: lesion depth (L) means (standard-deviation) after remineralization/staining cycling, and lesion depth change (ΔL) after second demineralization.

			L Lesion depth(µm)		ΔL Lesion depth (µm) [+]			
Groups			After cycling		Non-demin		Demin	
Remineralizing/Staining protocol	Non-Metallic	G1 (Control)	33.8 (12.7)	§	-1.0 (2.1)	A/a*	16.3 (18.9)	B/a
		G2 (At-home)	32.2 (9.2)	§	6.0 (11.0)	A/ab*	39.4 (17.1)	B/b
		G3 (In-office)	30.0 (6.4)	§	8.3 (7.6)	A/b*	19.3 (10.1)	B/a*
	Metallic	G4 (Control)	23.9 (5.4)	§	-2.9 (4.4)	A/a*	5.8 (4.5)	B/a
		G5 (At-home)	25.3 (4.2)	§	5.7 (6.0)	A/a*	9.1 (4.3)	A/a
		G6 (In-office)	24.7 (6.9)	§	5.2 (5.3)	A/a*	18.0 (10.6)	B/b*

Uppercase letters indicate significant difference within treatment (row, $p<0.05$); while lower case among treatments (column, $p<0.05$).

*: Indicate no significant difference between stain type (Non-metallic vs. Metallic).

§: Indicate significant difference between stain type.
+: ΔL =(L TMR bleaching/2nd demin – L TMR cycling).

References

1. Penumatsa NV, Kaminedi RR, Baroudi K and Barakath O (2015) Evaluation of remineralization capacity of casein phosphopeptide-amorphous calcium phosphate on the carbamide peroxide treated enamel. Journal of pharmacy & bioallied sciences 7:S583-6. doi: 10.4103/0975-7406.163556

2. Mastroberardino S, Campus G, Strohmenger L, Villa A and Cagetti MG (2012) An Innovative Approach to Treat Incisors Hypomineralization (MIH): A Combined Use of Casein Phosphopeptide-Amorphous Calcium Phosphate and Hydrogen Peroxide-A Case Report. Case reports in dentistry 2012:379593. doi: 10.1155/2012/379593

3. Walsh LJ and Brostek AM (2013) Minimum intervention dentistry principles and objectives. Australian dental journal 58 Suppl 1:3-16. doi: 10.1111/adj.12045

4. Carey CM (2014) Tooth whitening: what we now know. The journal of evidence-based dental practice 14 Suppl:70-6. doi: 10.1016/j.jebdp.2014.02.006

5. Cakir FY, Korkmaz Y, Firat E, Oztas SS and Gurgan S (2011) Chemical analysis of enamel and dentin following the application of three different at-home bleaching systems. Operative dentistry 36:529-36. doi: 10.2341/11-050-l

6. de Vasconcelos AA, Cunha AG, Borges BC, Vitoriano Jde O, Alves-Junior C, Machado CT and dos Santos AJ (2012) Enamel properties after tooth bleaching with hydrogen/carbamide peroxides in association with a CPP-ACP paste. Acta odontologica Scandinavica 70:337-43. doi: 10.3109/00016357.2011.654261

7. Bizhang M, Seemann R, Duve G, Romhild G, Altenburger JM, Jahn KR and Zimmer S (2006) Demineralization effects of 2 bleaching procedures on enamel surfaces with and

without post-treatment fluoride application. Operative dentistry 31:705-9. doi: 10.2341/05-144

8. Al-Angari SS and Hara AT (2016) A conservative approach to esthetically treat stained arrested caries lesions. Quintessence international (Berlin, Germany : 1985) 47:499-504. doi: 10.3290/j.qi.a36010

9. Lippert F, Lynch RJ, Eckert GJ, Kelly SA, Hara AT and Zero DT (2011) In situ fluoride response of caries lesions with different mineral distributions at baseline. Caries research 45:47-55. doi: 10.1159/000323846

10. Stookey GK, Burkhard TA and Schemehorn BR (1982) In vitro removal of stain with dentifrices. Journal of dental research 61:1236-9.

11. Yee R, Holmgren C, Mulder J, Lama D, Walker D and van Palenstein Helderman W (2009) Efficacy of silver diamine fluoride for Arresting Caries Treatment. Journal of dental research 88:644-7. doi: 10.1177/0022034509338671

12. Ando M, van Der Veen MH, Schemehorn BR and Stookey GK (2001) Comparative study to quantify demineralized enamel in deciduous and permanent teeth using laser- and light-induced fluorescence techniques. Caries research 35:464-70.

13. Johnston WM and Kao EC (1989) Assessment of appearance match by visual observation and clinical colorimetry. Journal of dental research 68:819-22.

14. Alqahtani MQ (2014) Tooth-bleaching procedures and their controversial effects: A literature review. The Saudi Dental Journal 26:33-46. doi: 10.1016/j.sdentj.2014.02.002

15. Cortes G, Pini NP, Lima DA, Liporoni PC, Munin E, Ambrosano GM, Aguiar FH and Lovadino JR (2013) Influence of coffee and red wine on tooth color during and after

bleaching. Acta odontologica Scandinavica 71:1475-80. doi: 10.3109/00016357.2013.771404

16. Sulieman M, MacDonald E, Rees JS, Newcombe RG and Addy M (2006) Tooth bleaching by different concentrations of carbamide peroxide and hydrogen peroxide whitening strips: an in vitro study. Journal of esthetic and restorative dentistry : official publication of the American Academy of Esthetic Dentistry [et al] 18:93-100; discussion 101.

17. Meireles SS, Fontes ST, Coimbra LA, Della Bona A and Demarco FF (2012) Effectiveness of different carbamide peroxide concentrations used for tooth bleaching: an in vitro study. Journal of applied oral science : revista FOB 20:186- 91.

18. Matis BA, Cochran MA, Franco M, Al-Ammar W, Eckert GJ and Stropes M (2007) Eight in-office tooth whitening systems evaluated in vivo: a pilot study. Operative dentistry 32:322-7. doi: 10.2341/06-135

19. Zekonis R, Matis BA, Cochran MA, Al Shetri SE, Eckert GJ and Carlson TJ (2003) Clinical evaluation of in-office and at-home bleaching treatments. Operative dentistry 28:114-21.

20. Jones AH, Diaz-Arnold AM, Vargas MA and Cobb DS (1999) Colorimetric assessment of laser and home bleaching techniques. Journal of esthetic dentistry 11:87-94.

21. Mielczarek A, Klukowska M, Ganowicz M, Kwiatkowska A and Kwasny M (2008) The effect of strip, tray and office peroxide bleaching systems on enamel surfaces in vitro. Dental materials : official publication of the Academy of Dental Materials 24:1495-500. doi: 10.1016/j.dental.2008.03.009

22. Miranda CB, Pagani C, Benetti AR and Matuda Fda S (2005) Evaluation of the bleached human enamel by Scanning Electron Microscopy. Journal of applied oral science : revista FOB 13:204-11.

23. Soares DG, Ribeiro AP, Sacono NT, Loguercio AD, Hebling J and Costa CA (2013) Mineral loss and morphological changes in dental enamel induced by a 16% carbamide peroxide bleaching gel. Brazilian dental journal 24:517-21. doi: 10.1590/0103-6440201302225

24. Sun L, Liang S, Sa Y, Wang Z, Ma X, Jiang T and Wang Y (2011) Surface alteration of human tooth enamel subjected to acidic and neutral 30% hydrogen peroxide. Journal of dentistry 39:686-92. doi: 10.1016/j.jdent.2011.07.011

25. Sa Y, Sun L, Wang Z, Ma X, Liang S, Xing W, Jiang T and Wang Y (2013) Effects of two in-office bleaching agents with different pH on the structure of human enamel: an in situ and in vitro study. Operative dentistry 38:100-10. doi: 10.2341/11-173-l

26. Turssi CP, Messias DF, Corona SM and Serra MC (2010) Viability of using enamel and dentin from bovine origin as a substitute for human counterparts in an intraoral erosion model. Brazilian dental journal 21:332-6.

27. Silva AF, Demarco FF, Meereis CT, Cenci MS and Piva E (2014) Light-activated bleaching: effects on surface mineral change on enamel. The journal of contemporary dental practice 15:567-72.

28. Salomao D, Santos D, Nogueira R, Palma-Dibb R and Geraldo-Martins V (2014) Acid demineralization susceptibility of dental enamel submitted to different bleaching techniques and fluoridation regimens. Operative dentistry 39:E178-85. doi: 10.2341/13-140

29. Yu DG, Kimura Y, Fujita A, Hossain M, Kinoshita JI, Suzuki N and Matsumoto K (2001) Study on acid resistance of human dental enamel and dentin irradiated by semiconductor laser with Ag(NH3)2F solution. Journal of clinical laser medicine & surgery 19:141-6. doi: 10.1089/10445470152927973

GENERAL DISCUSSION

Due to differences on the treatment philosophies of stained arrested caries lesions (s-ACLs), whether to restore or not, it is reasonable to assume that the diagnosis and treatment decision associated with these lesions can be misleading for some dentists [12]. Therefore, some s-ACLs may be unnecessarily restored to improve their aesthetics [9].

Bleaching agents improve the color lightness of s-ACLs, making them gradually blend with the surrounding tooth, improving its aesthetics. Our research proposed a novel conservative approach to aesthetically treat s-ACLs, verifying its efficacy and potential safety concerns. Two commonly used dental bleaching protocols were tested, namely in-office and at-home bleaching.

The 1st paper (chapter 1) proposed the use of bleaching systems to conservatively improve the aesthetics of ACLs. The potential of this novel approach was shown both in-vitro and clinically. The promising results led us to further investigate the physical properties related to this approach, by developing stained remineralized caries-like lesion (s-RCL) model (chapter 2) to facilitate testing the bleaching treatment efficacy and safety in-vitro. The proposed model artificially created remineralized (saliva, fluoride) and stained (non-metallic and metallic) lesions.

In chapters 2 and 3, we exposed artificial caries lesion to two clinically common stain types during cycling. These tested stains were used to clinically characterize a non-

metallic model (orange/brown stains) related to organic chromogens (dietary stains, tobacco, etc.) and metallic model (black stains), found in stains from medicines (containing iron supplements, metal salts, etc.) and cationic antiseptics. These different cycling groups (non-metallic and metallic) were designed to mimic the dynamic variations in stains and mineral saturation (remineralization) as to better simulate different clinical situation of s-ACLs.

We observed that different stain types had different responses to bleaching treatment. In the 3rd paper (chapter 3), we studied the efficacy of different bleaching protocols (simulating at-home/15% carbamide peroxide and in-office/40% hydrogen peroxide) on these artificially created lesions, along with its safety by evaluating the model susceptibility to further demineralization after bleaching.

Based on the wealthy of knowledge in the literature discussing safety of dental bleaching procedures, our studies (chapter 1,2 and 3) have shown that both bleaching systems improved the aesthetics of s-ACLs, with efficacy being dependent on the nature of the stain and bleaching agent.

In general, at-home bleaching protocol presented greater efficacy in color improvement especially in the non-metallic model (organic stains), yet enamel was more susceptible to subsequent demineralization compared to in-office systems. This limitation might be compensated clinically by the remineralization action of saliva, as this fluid presents buffering capacity and is supersaturated in calcium and phosphate ions [13]. Topical

fluoride application is another measure that can enhance the remineralization process [14]. This understanding will allow clinicians to carefully consider selecting appropriate cases, based on stain types, and bleaching systems, to produce optimum clinical satisfactory result with combined safety and efficacy.

Bleaching ACLs clinically would serve as a non-invasive, effective, safe, predictable and inexpensive approach to improve the aesthetics of ACLs [11]. Despite these benefits, bleaching has been reported to cause tooth sensitivity [15], and increase tooth surface roughness possibly making it more prone for plaque accumulation [16]. Further speculating, bleaching stained lesions may also negatively affect the capability of caries detection methods to differentiate sound from affected enamel (brown spot/ white spot lesions), especially those based on light excitation wavelengths differentiation such as fluorescence spectroscopy [17].

Also, it may have a potential implication on the use of ICDAS as a tool to monitor the progression/arrest of caries lesions. Moreover, in some cases bleaching may not result in a complete elimination of the discoloration and would require additional surgical intervention. However, there is still a benefit as the amount of tissue removal after bleaching would be less extensive resulting in a restoration with a minimum thickness. Finally, clinicians should carefully consider immediate resin bonding to the bleached surface. When the bleaching agent dissociates into free radicals [18] it forms an oxygen inhibiting layer, which potentially interferes with resin polymerization and subsequently reduces the restoration bonding strength [19, 20]. This is an important topic for further

consideration, by evaluating the bond strength between the bleached surface of such created lesions and restorations.

The simulated s-ACLs created in our study allowed us to further investigate mechanistic aspects related to the efficacy and safety of different bleaching protocols of these lesions, in a quick, cost effective and systematic fashion. Other applications would include using these lesions in laboratory as screening tests for different bleaching agents and protocols.

Additionally, in-vitro tests have the advantage of carrying out different variables investigated under highly controlled conditions compared to in-vivo. On the other hand, the significant limitation of these models is their inability to simulate the complex biological processes involved in creating stained ACL's. This includes the continuous presence of oral bacteria, saliva, antimicrobial enzymes and proteins, different organic and non-organic stains all of which may affect the s-RCL model development.
There is a need for more clinical data to support bleaching as aesthetic treatment for s-ACLs, investigating different bleaching systems, concentrations and different stain types.

GENERAL CONCLUSION

Overall, bleaching can improve the aesthetics of stained arrested caries lesions; however it depends on the type of staining involved, as well as on the bleaching system used. The presented *in vitro* experimental models were able to create simulated stained-remineralized-caries-like lesions.

Non-metallic stains were lighter in color and more responsive to bleaching treatment compared to metallic stains. The at-home bleaching protocol presented greater efficacy compared to in-office. However, it was associated with increased susceptibility to subsequent demineralization especially in non-metallic stained lesions.

APPENDICES

Appendix 1: Color change ANOVA comparison of two-way and three-way interactions among stain type, substrate condition (demineralized or sound) and time points in enamel.

Effect	NumDF	DenDF	FValue	ProbF	Sig
Stain type	3	260	163.18	<.0001	*
Condition	1	260	160.11	<.0001	*
Time	2	260	108.49	<.0001	*
Stain type *Condition	3	260	54.11	<.0001	*
Stain type *Time	6	260	195.64	<.0001	*
Condition*Time	2	260	27.86	<.0001	*
Stain type *Condition*Time	6	260	26.50	<.0001	*

Appendix 2: Color change ANOVA comparison of two-way and three-way interactions among stain type, substrate condition (demineralized or sound) and time points in dentin.

Effect	NumDF	DenDF	FValue	ProbF	Sig
Stain type	3	260	305.51	<.0001	*
Condition	1	260	88.31	<.0001	*
Time	2	260	1169.61	<.0001	*
Stain type *Condition	3	260	49.63	<.0001	*
Stain_type*Time	6	260	733.46	<.0001	*
Condition*Time	2	260	24.52	<.0001	*
Stain type *Condition*Time	6	260	29.60	<.0001	*

Appendix 3: Mineral loss ANOVA comparison of the interaction between stain type and substrate condition (demineralized or sound) in enamel.

Effect	NumDF	DenDF	FValue	ProbF
Stain type	3	52	17.82	<.0001
Condition	1	52	421.93	<.0001
Stain type *Condition	3	52	15.59	<.0001

Appendix 4: Lesion depth ANOVA comparison of the interaction between stain type and substrate condition (demineralized or sound) in enamel.

Effect	NumDF	DenDF	FValue	ProbF
Stain type	3	52	16.70	<.0001
Condition	1	52	451.41	<.0001
Stain type *Condition	3	52	12.15	<.0001

Appendix 5: Mineral loss ANOVA comparison of the interaction between stain type and substrate condition (demineralized or sound) in dentin.

Effect	NumDF	DenDF	FValue	ProbF
Stain type	3	52	44.71	<.0001
Condition	1	52	1059.27	<.0001
Stain type *Condition	3	52	20.11	<.0001

Appendix 6: Lesion depth ANOVA comparison of the interaction between stain type and substrate condition (demineralized or sound) in dentin.

Effect	NumDF	DenDF	FValue	ProbF
Stain type	3	52	36.96	<.0001
Condition	1	52	1340.98	<.0001
Stain type *Condition	3	52	11.42	<.0001

Appendix 7: Color change ANOVA comparison of two-way, three-way and four-way interactions among stain type, bleach, 2nd demineralization and time points.

Effect	NumDF	DenDF	FValue	ProbF	Sig
Stain type	1	114	933.14	<.0001	*
Bleach	2	114	43.73	<.0001	*
2nd demin	1	114	0.12	0.7314	
Time	2	114	4741.70	<.0001	*
Stain type*Bleach	2	114	0.76	0.4682	
Stain type*2nd demin	1	114	0.04	0.8501	
Stain type*Time	2	114	1676.61	<.0001	*
Bleach*2nd demin	2	114	0.82	0.4414	
Bleach*Time	4	114	48.24	<.0001	*
Time*2nd demin	2	114	0.73	0.4826	
Stain type *Bleach*2nd demin	2	114	1.79	0.1709	
Stain type *Bleach*Time	4	114	1.31	0.2695	
Stain type *Time*2nd demin	2	114	0.96	0.3858	

Bleach*Time*2nd demin	4	114	3.07	0.0192	*
Stain type*Bleach*Time*2nd	4	114	2.22	0.0712	

Appendix 8: Mineral loss (after cycling and 2nd demineralization) ANOVA comparison of two-way, three-way and four-way interactions among stain type, bleach, 2nd demineralization and time points.

Effect	NumDF	DenDF	FValue	ProbF	Sig
Stain type	1	114	29.14	<.0001	*
Bleach	2	114	9.83	0.0001	*
2nd demin	1	114	21.50	<.0001	*
Time	1	114	278.08	<.0001	*
Stain type*Bleach	2	114	0.84	0.4336	
Stain type*2nd demin	1	114	4.47	0.0367	*
Stain type*Time	1	114	4.53	0.0355	*
Bleach*2nd demin	2	114	2.90	0.0591	
Bleach*Time	2	114	23.69	<.0001	*
Time*2nd demin	1	114	85.83	<.0001	*
Stain type*Bleach*2nd demin	2	114	1.88	0.1580	
Stain type*Bleach*Time	2	114	1.59	0.2085	
Stain type*Time*2nd demin	1	114	4.13	0.0445	*
Bleach*Time*2nd demin	2	114	1.91	0.1521	
Stain type*Bleach*Time*2nd demin	2	114	1.22	0.2980	

Appendix 9: Mineral loss (ΔΔZ) ANOVA comparison of the interaction of two-way and three-way among stain type, bleach and 2nd demineralization.

Effect	NumDF	DenDF	FValue	ProbF	Sig
Stain type	1	114	4.53	0.0355	*
Bleach	2	114	23.69	<.0001	*
Demin2	1	114	85.83	<.0001	*
Stain type*Bleach	2	114	1.59	0.2085	
Stain type*2nd	1	114	4.13	0.0445	*
Bleach*2nd demin	2	114	1.91	0.1521	
Stain type*Bleach*2nd demin	2	114	1.22	0.2980	

Appendix 10: Lesion depth (after cycling and 2nd demineralization) ANOVA comparison of the interaction of two-way, three-way and four-way among stain type, bleach, 2nd demineralization and time points.

Effect	NumDF	DenDF	FValue	ProbF	sig
Stain type	1	114	100.74	<.0001	*
Bleach	2	114	7.27	0.0011	*
2nd demin	1	114	55.80	<.0001	*
Time	1	114	145.41	<.0001	*
Stain type*Bleach	2	114	5.20	0.0069	*
Stain type*2nd demin	1	114	35.09	<.0001	*
Stain type*Time	1	114	19.60	<.0001	*
Bleach*2nd demin	2	114	3.99	0.0212	*
Bleach*Time	2	114	12.70	<.0001	*
Time*2nd demin	1	114	65.45	<.0001	*
Stain type*Bleach*2nd demin	2	114	3.06	0.0508	
Stain type *Bleach*Time	2	114	4.79	0.0100	*
Stain type *Time*2nd demin	1	114	11.85	0.0008	*
Bleach*Time*2nd demin	2	114	1.26	0.2869	
Stain type *Bleach*Time*2nd demin	2	114	6.86	0.0015	*

Appendix 11: Lesion depth (ΔΔL) ANOVA comparison of the interaction of two- way and three-way among stain type, bleach and 2nd demineralization.

Effect	NumDF	DenDF	FValue	ProbF	Sig
Stain type	1	114	19.60	<.0001	*
Bleach	2	114	12.70	<.0001	*
2nd demin	1	114	65.45	<.0001	*
Stain type*Bleach	2	114	4.79	0.0100	*
Stain type*2nd demin	1	114	11.85	0.0008	*
Bleach*2nd demin	2	114	1.26	0.2869	
Stain type*Bleach*2nd demin	2	114	6.86	0.0015	*

Appendix 12: Color coordinates (ΔL, Δa, Δb) means after demineralization, staining, and bleaching.

Groups	ΔE_{Demin}^{+}			$\Delta E_{Cycling}^{+}$			$\Delta E_{Bleaching}^{+}$		
	L	a	b	L	a	b	L	a	b
G1 (control)	72.1	-2.2	3.4	54.5	-0.5	7.2	54.5	-0.7	9.4
G2 (At-home)	71.3	-1.9	5.3	55.4	-0.1	8.4	73.6	-2.9	-1.0
G3 (In-office)	70.0	-1.7	4.7	53.5	-0.1	8.0	67.7	-2.9	0.1

G4 (control)	71.0	-1.7	3.7	13.3	4.8	5.5	18.3	4.7	7.1
G5 (At-home)	70.6	-1.6	3.6	12.9	5.2	6.1	40.9	4.9	0.5
G6 (In-office)	71.2	-2.0	6.0	12.9	4.3	4.3	34.7	5.5	6.0

Figure A.1. Photograph of two specimens after demineralization. A: Dentin slab, B: Enamel slab. The right side of each specimen represents the demineralized surface while the left side represents the sound surface.

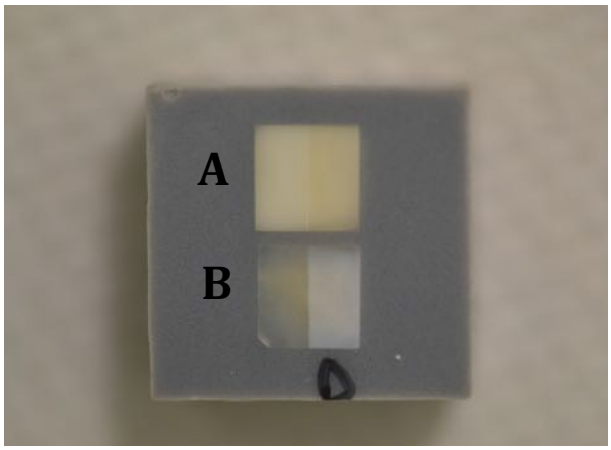


Figure A.2. Photograph of the cycling machine. A: Rotating rod carrying specimens, B: Metallic staining solution.

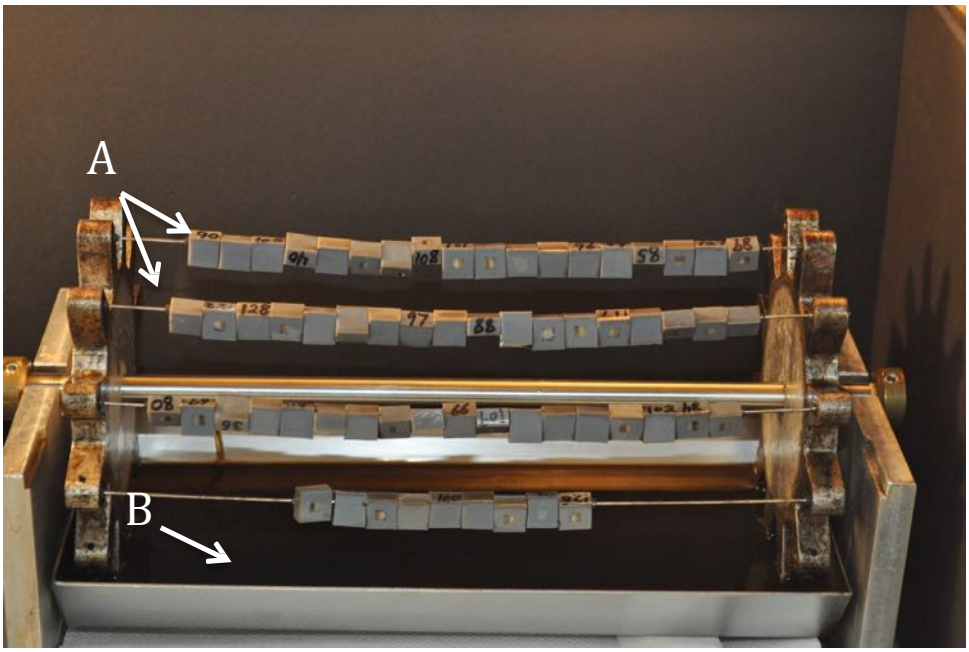
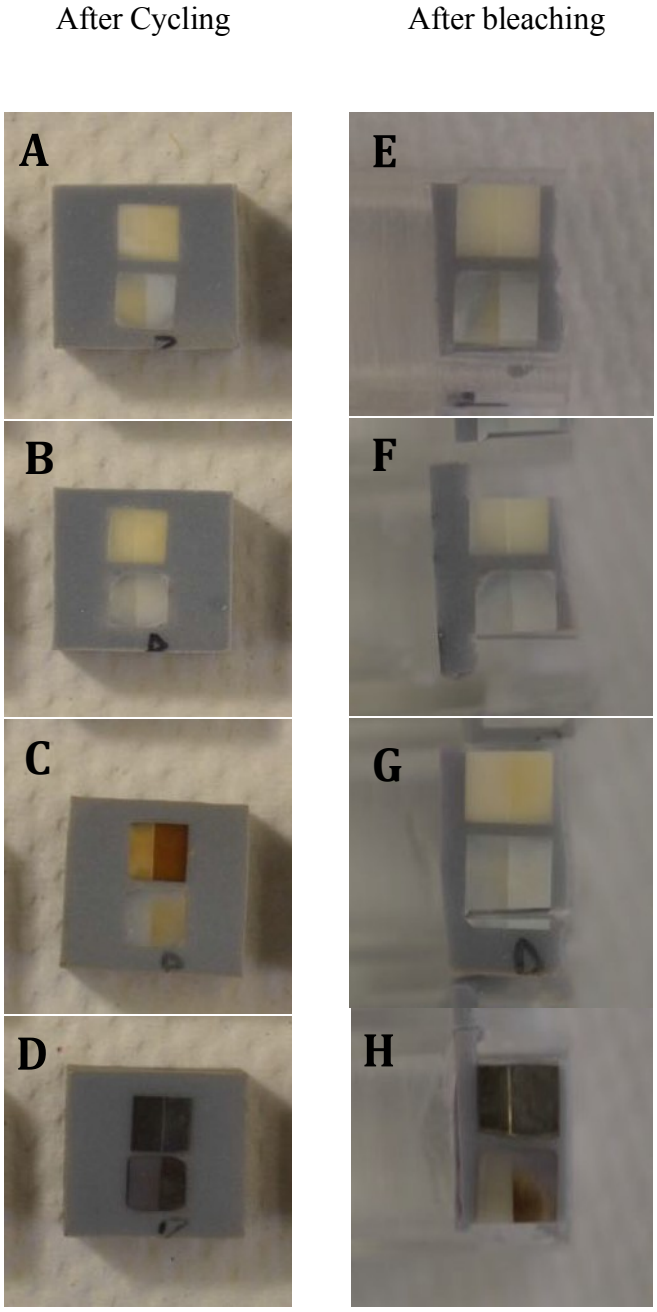


Figure A.3. Photographs of the specimens, after cycling (left) and after bleaching (right). A/E: Negative control, B/F: Remin-control, C/G: Non-metallic stain, D/H: Metallic stain.



REFERENCES

1. Ahmad I (2010) Risk management in clinical practice. Part 5. Ethical considerations for dental enhancement procedures. British dental journal 209:207- 14. doi: 10.1038/sj.bdj.2010.769

2. Demarco FF, Meireles SS and Masotti AS (2009) Over-the-counter whitening agents: a concise review. Brazilian oral research 23 Suppl 1:64-70.

3. Penumatsa NV, Kaminedi RR, Baroudi K and Barakath O (2015) Evaluation of remineralization capacity of casein phosphopeptide-amorphous calcium phosphate on the carbamide peroxide treated enamel. Journal of pharmacy & bioallied sciences 7:S583-6. doi: 10.4103/0975-7406.163556

4. Jefferies SR (2014) Advances in remineralization for early carious lesions: a comprehensive review. Compendium of continuing education in dentistry (Jamesburg, NJ : 1995) 35:237-43; quiz 244.

5. Karlinsey RL, Mackey AC, Dodge LE and Schwandt CS (2014) Noncontact remineralization of incipient lesions treated with a 5% sodium fluoride varnish in vitro. Journal of dentistry for children (Chicago, Ill) 81:7-13.

6. Horst JA, Ellenikiotis H and Milgrom PL (2016) UCSF Protocol for Caries Arrest Using Silver Diamine Fluoride: Rationale, Indications and Consent. Journal of the California Dental Association 44:16-28.

7. Watts A and Addy M (2001) Tooth discolouration and staining: a review of the literature. British dental journal 190:309-16. doi: 10.1038/sj.bdj.4800959a

8. Anfe TE, Agra CM and Vieira GF (2011) Evaluation of the possibility of removing staining by repolishing composite resins submitted to artificial aging.

Journal of esthetic and restorative dentistry : official publication of the American Academy of Esthetic Dentistry [et al] 23:260-7. doi: 10.1111/j.1708- 8240.2011.00435.x

9. Holmgren C, Gaucher C, Decerle N and Domejean S (2014) Minimal intervention dentistry II: part 3. Management of non-cavitated (initial) occlusal caries lesions-- non-invasive approaches through remineralisation and therapeutic sealants. British dental journal 216:237-43. doi: 10.1038/sj.bdj.2014.147

10. Kelleher MG, Djemal S and Lewis N (2012) Ethical marketing in'aesthetic' ('esthetic') or'cosmetic dentistry'. Part 1. Dental update 39:313-6, 318-20, 323-4 passim.

11. Sundfeld RH, Neto DS, Machado LS, de Oliveira FG, de Alexandre RS, Palo RM and Sundefeld ML (2014) Dental bleaching with a 10% hydrogen peroxide product: A six-month clinical observation. Indian journal of dental research : official publication of Indian Society for Dental Research 25:4-8. doi: 10.4103/0970-9290.131046

12. Lee RC, Staninec M, Le O and Fried D (2016) Infrared Methods for Assessment of the Activity of Natural Enamel Caries Lesions. IEEE journal of selected topics in quantum electronics : a publication of the IEEE Lasers and Electro-optics Society 22. doi: 10.1109/jstqe.2016.2542481

13. Smidt A, Feuerstein O and Topel M (2011) Mechanical, morphologic, and chemical effects of carbamide peroxide bleaching agents on human enamel in situ. Quintessence international (Berlin, Germany : 1985) 42:407-12.

14. Arif N, Beighton D and Sheehy EC (2006) Microbiological assessment of occlusal brown-spot lesions in primary molars. Caries research 40:398-402. doi: 10.1159/000094285

15. Loguercio AD, Tay LY, Herrera DR, Bauer J and Reis A (2015) Effectiveness of nano-calcium phosphate paste on sensitivity during and after bleaching: a randomized

clinical trial. Brazilian oral research 29:1-7. doi: 10.1590/1807- 3107BOR-2015.vol29.0099

16. Melo CF, Manfroi FB and Spohr AM (2014) Microhardness and roughness of enamel bleached with 10% carbamide peroxide and brushed with different toothpastes: an in situ study. Journal of international oral health : JIOH 6:18-24.

17. Buchalla W (2005) Comparative fluorescence spectroscopy shows differences in noncavitated enamel lesions. Caries research 39:150-6. doi: 10.1159/000083162

18. Alqahtani MQ (2014) Tooth-bleaching procedures and their controversial effects: A literature review. The Saudi Dental Journal 26:33-46. doi: 10.1016/j.sdentj.2014.02.002

19. Dishman MV, Covey DA and Baughan LW (1994) The effects of peroxide bleaching on composite to enamel bond strength. Dental materials : official publication of the Academy of Dental Materials 10:33-6.

20. Moon HJ (2015) Treatment after bleaching for optimal bonding. Restorative dentistry & endodontics 40:177-8. doi: 10.5395/rde.2015.40.2.177

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