The Use of Natural Plant Extracts to Enhance Caries Prevention and to Improve the Performance of Dental Adhesives

"Role of natural extracts to achieve super tooth"

Dr. Md. Sofiqul Islam

Promoter: Professor Junji Tagami

Cariology and Operative Dentistry

Department of Oral Health Sciences

Graduate School, Tokyo Medical and Dental University

Thesis presented for the degree of Doctor of Philosophy in Dental Sciences

Tokyo, Japan. 2014

Dedication

To my late father Md. Akber Ali, my mother Mrs. Morjina Begum, my brother Md. Rafiqul Islam, my sister in law Mrs. Tamima Akter and most importantly my wife Dr. Smriti Aryal A. C for their help and encouragement over the years.

Acknowledgments

The works presented in this thesis were carried out at Cariology and Operative Dentistry, Oral Restitution Department, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University (TMDU) from 2010 to 2014, and were supported by the Global Center of Excellence (GCOE), International Research Center for Molecular Science in Tooth and Bone Diseases at TMDU. This thesis and the degree of PhD are the outcome of the efforts and assistance of several people from different universities and organizations. I would like to express my deepest gratitude to all who contributed to this final product and to those who are not mentioned below.

Firstly, I would like to express my sincere gratitude to Professor **Junji Tagami**, Professor and Chairman of Cariology and Operative Dentistry Department, TMDU, who kindly provided me the opportunity and facilities to undertake this study, for sharing his immense knowledge of research and life and for his unbound support. Secondly my gratitude and appreciation to my research group supervisor, Dr. **Masayuki Otsuki**, who has supported me throughout my work with his patience and knowledge whilst allowing me the room to work in my own way. I wish to acknowledge my sincere gratitude to my direct supervisor, Dr. **Noriko Hiraishi**, for all her generosity in teaching and her constant supportive attitude towards my work. Her guidance and scientific experiences were essential for the development, process and conclusion of the studies herein. Without her this thesis would not have been completed or written. I am deeply grateful to another co-worker of my research group Dr. **Mohannad Nassar** and Dr. **Kong Kalyan** for their valuable support in an experiment and paper work. The academic staff of Cariology and Operative Dentistry, Oral Restitution Department, especially, Dr. Toru Nikaido, Dr. Masatoshi Nakajima, Dr. Yasushi Shimada, Dr.

Takako Yoshikawa, Dr. Yuichi Kitasako, Dr. Go Inoue, Dr. Keiichi Hosaka, Dr. Tomohiro Takagaki, Dr. Rena Takahashi, Dr. Masahiro Takahashi, Dr. Hidenori Hamba, Dr. Alireza Sadr and Dr. Syozi Nakashima for their helpful instructions and comments. I owe my deepest gratitude to Dr. Shizuko Ichinose of the Instrumental Analysis Research Center for providing useful information and to allow me to use their facilities. My sincere thanks to my former colleagues at TMDU, for sharing unforgettable memories and helping me during my graduate studies; Dr. Ahmed Bakry, Dr. Patricia Makishi, Dr. Sitthikorn Kunawarote, Dr. Hamid Nurrohman, Dr. Amir Nazari, Dr Ilnaz Hariri, Dr. Taweesak Prasansuttiporn, Dr. Sachiko Utaka, Dr. Turki Bakhsh, Dr. Suppason Thitthaweerat, and to my current colleagues; Dr. Mona Mandurah, Dr. Gerardo Joves Mendez, Dr. Aidil Akmal, Dr. Ena Lodha, Dr. Ornnicha Thanatvarakorn, Dr. Alaa Turkistani, Dr. Sahar Khunkar, Dr. Ehab Al-Sayed, Dr. Teerapong Mamanee, Dr. Baba Bista, Dr. Maria Jacinta Rosario Romero, Dr. Patrycja Majkut. A special word of thanks goes to my collaborators, Professor Cynthia Yiu, Dr. Yukihiko Tamura, Professor Shouhei Kasugai, Professor Keiichi Ohya, Dr. Shu Taira, Professor Daisaku Kaneko, Mr. Ryohei Sono and Mr.Tsutomu Takatsura. My deep-felt appreciation goes to R&D Center Sunstar Inc. Japan and other manufacturing companies to support my work with their material and research facilities. The academics and former colleagues at Bangabandhu Sheikh Mujib Medical University, Dhaka, Bangladesh and Pioneer Dental College, Dhaka, Bangladesh. Lastly, I would like to thank my family for all their love and encouragement. And most of all, important thanks goes to my wife Dr. Smriti Aryal A.C for the love, support and constant contribution to overcome the challenges of life. It is so wonderful to have you beside me in the past, present and future.

This thesis is based on the original research works by the author, to which the following articles refer.

Chapter 1	Introduction
Chapter 2	Islam SM, Hiraishi N, Nassar M, Sono R, Otsuki M, Takatsura T, Yiu C, Tagami J. In vitro effect of hesperidin on root dentin collagen and de/re-mineralization. Dent Mater J. 2012; 31(3):362-7.
Chapter 3	Islam S, Hiraishi N, Nassar M, Yiu C, Otsuki M, Tagami J. Effect of natural cross-linkers incorporation in a self-etching primer on dentine bond strength. J Dent. 2012 Dec; 40 (12): 1052-9.
Chapter 4	Islam S, Hiraishi N, Nassar M, Yiu C, Otsuki M, Tagami J. Effect of Hesperidin Incorporation into a Self-Etching Primer on Durability of Dentin Bond. (DEMA-D-13-00137)
Chapter 5	General discussion and conclusion
Chapter 6	References
Chapter 7	Biography

Table of Content

Abstract

The Use of Natural Plant Extracts to Enhance Caries Prevention and to Improve the Performance of Dental Adhesives

Introduction: Natural plant extracts have been used as folk medicine for thousands of years and are promising sources for novel therapeutic agents. They have been focused on by several recent studies as potential materials to prevent oral diseases such as dental caries. In our consecutive studies, we have attempted to find the possible role of natural extracts to modify and reinforce natural teeth. Natural extract-derived flavonoids such as hesperidin (HPN) and proanthocyanidins (PA) are expected to work as natural collagen cross-linkers. Reinforcement of both the collagen matrix and inorganic apatite are important in caries prevention. For durable dentin bonding, resin-dentin interfaces must be preserved from biodegradation. In our studies, we have attempted to (1) investigate the effects of HPN on human root dentin demineralization and collagen preservation and compare it with chlorhexidine (CHX) and PA; (2) investigate the effect of incorporation of natural cross-linkers into the primer of a self-etching adhesive on the immediate micro-tensile bond strength (μTBS) and (3) evaluate the effects of incorporation of natural cross-linkers into a self-etch adhesive primer on the long-term μTBS to dentin.

Materials and methods: (1) Human root dentin blocks were assigned to different treatment groups: 0.5% HPN, 0.5% CHX and 0.5% PA. Specimens were subjected to pH cycling by demineralization for 14h, incubation in testing solutions for 2h and 8h re-mineralization in the presence of bacteria-derived collagenase for 8 days. Calcium release was measured by means of an atomic absorption spectrophotometer and degraded collagen matrix was investigated by

hydroxyproline assay. Specimens were assessed longitudinally with transverse micro-radiography to investigate the lesion depth and mineral loss. (2) Flat dentin surfaces were prepared from extracted human molar teeth. The 0.5% HPN, 0.5% CHX or 0.5% PA was incorporated into Clearfil SE primer (Kuraray Noritake Dental Inc.) to formulate three experimental primers. The original SE primer served as control. Following these primer application, the teeth were bonded with Clearfil SE bond, restored with resin composite and stored in water for 24 hours at 37ºC. The bonded specimens were sectioned into beams and subjected to micro tensile bond testing (μTBS). Failure analysis and morphological evaluation of the dentin surfaces were performed using a scanning electron microscope (SEM). Hardness (H) and elastic modulus (EM) were measured using a nano-indentation test to examine the mechanical properties of the bonded interfaces. (3) The experimental primers were prepared by incorporating 0.5%, 1%, 2%, 5% of HPN or 0.5% of PA into Clearfil SE primer. Extracted human molar teeth were restored using the experimental primers or the pure SE primer (control). The mechanical properties of the bonded interfaces were measured using the nano-indentation test. Beam-shaped bonded specimens were sub-divided for one-day and one-year μTBS test. Interfacial collagen morphology was observed using transmission electron microscopy.

Results: (1) In 0.5% HPN and 0.5% PA groups, demineralization was reduced when the collagen matrix was preserved. The hesperidin group showed the lowest value in lesion depth and mineral loss. (2) The μTBS, H and EM showed significant differences among the tested and control groups. Multiple comparison revealed that incorporation of HPN significantly increased μTBS, H and EM, when compared with the other groups ($p<0.006$). The PA-incorporated group significantly decreased μTBS, H and EM, when compared with the other groups;

while CHX-incorporated group did not show any statistical significant difference when compared with the control group. (3) The immediate μTBS significantly increased in 0.5%, 1% and 2% HPN-incorporated groups when compared with the control. The immediate mechanical properties of the bonded interface were improved by 1% and 2% HPN-incorporated primers. For the long-term μTBS, the 2% and 5% HPN-incorporated groups were significantly higher than the control. The morphology of the collagen fibrils was preserved by 5% HPN-incorporation after one-year storage. The PA group, however, failed to improve both the immediate and long term μTBS and the mechanical properties of the bonded interfaces.

Significance: (1) Hesperidin inhibited demineralization and probably enhanced re-mineralization even under fluoride-free conditions. (2) Incorporation of HPN into Clearfil SE primer had a positive influence on the immediate μTBS and mechanical properties of the bonded interface. (3) The incorporation of 2% HPN into the self-etching primer had a positive effect on the immediate μTBS and mechanical properties of the resin-dentin interface. The 5% HPN group preserved the morphology of the collagen in the hybrid layer after one-year storage in the artificial saliva.

Japanese summary

天然抽出フラボノイドによる、(1)う蝕抑制（象牙質コラーゲン保護と脱灰象牙質の再石灰効果）と(2) 象牙質接着性向上への効果

序論: 再石灰化は i)う蝕予防の一部として、歯を脱灰から守る本来の保護機能を助長させるもの、ii) う蝕治療の一部として、Minimal Intervention 治療、つまり歯を削らない「最小限の侵襲」で接着性材料を使い、う蝕歯を保存する試みに応用が期待されている。歯質象牙質は 70%が無機質（ヒドロキシアパタイト）、20%が有機物（膠原繊維（コラーゲン繊維）と非膠原性タンパク質）からなり、う蝕のメカニズムを考える上で、酸による無機質の脱灰抑制とあわせて、有機質の保護を考慮しなければならない。近年、有機質の保護が確立できれば、これを足場にしてう蝕脱灰部の再石灰化が有意に起こると考えられている。フラボノイド (flavonoid) は天然に存在する安全性の高い有機化合物群で, 少量でも抗酸化作用を有し、天然の架橋剤としてコラーゲンを保護すると報告されている。本研究では天然フラボノイドの柑橘系由来ヘスペリジン及び、グレープシード抽出物プロアントシアニジンを歯質象牙質に作用させ、う蝕の進行への影響を評価した。　また、フラボノイドの架橋効果は、歯科接着修復学にも応用が期待される。象牙質は有機質を含むため、有機質の経時的劣化が接着力の低下に影響を及ぼしているため、有機質の保護強化が現在の課題である。よってヘスペリジンの抗酸化作用、天然の架橋剤として作用を利用し、歯科修復材の前処理剤として応用させる試みを行った。研究項目は以下三課題である。（１）ヘスペリジンによるヒト歯根面象牙質脱灰後のコラーゲンの保護と、再石灰化への影響と、プロアントシアニジンおよびクロルヘキシジン（コラーゲン分解酵素阻害剤）との比較評価。　（２）ヒト象牙質を使った、ヘスペリジン配合セルフエッチングプライマーの接着界面の物

性の向上、及び短期象牙質接着性への影響。（３）ヘスペリジン配合セルフエッチングプライマーの長期象牙質接着性への影響、接着界面劣化抑制効果の透過型電子顕微鏡観察。

(1)

方法: 本研究では天然フラボノイドのヘスペリジンを歯質象牙質に作用させ、う蝕の進行への影響を評価した。象牙質う蝕モデルとしてｐH を調整した脱灰、再石灰溶液にて 8 日間保存した。この間、象牙質にヘスペリジンン 0.5%溶液にて一日 2 時間作用させた。象牙質コラーゲンの、脱灰または再石灰化への影響を検討するために、再石灰化液中に Clostridium histolyticum 由来のコラゲナーゼを 7.5 U/ mL 作用させた。脱灰抑制効果評価は、脱灰液中溶解カルシウムを原子吸光分析にて定量を行った。象牙質コラーゲンの崩壊抑制効果の評価は、再石灰化液中の可溶性コラーゲンを加水分解後、アミノ酸分析を行った。さらに X 線を用いたミネラルプロファイル測定による脱灰部の分析を行った。同じフラボノイドのグレープシード抽出物プロアントシアニジン 0.5%と、う蝕予防に使用されるクロロヘキシジ 0.5%ンとの効果を比較検討した。

結果考察：ヘスペリジン群は有意に脱灰量、コラーゲン崩壊度を抑制していた。脱灰部の分析を行った結果、表層のみならず、やや深部まで再石灰化が見られた。これらの結果より、ヘスペリジンは、う蝕の脱灰抑制、再石灰化促成に何らかの効果があることが分かった。作用メカニズムは、今後さらなる研究が必要だが、う蝕の発生また進行をコントロールするうえで、生体に安全なフラボノイドは有効な薬理作用を持つと予測され、将来の治療薬と期待される。

（２）

方法: 本研究では、天然フラボノイドを市販の歯科接着材プライマーに配合させ、ヒト象牙質への接着性を評価した。クラレノリタケ社製クリアフ

ィル メガボンドプライマー（前処理剤）にヘスペリジン 0.5%を配合させた。ヒト歯質象牙質を使い、歯科接着剤で接着後、37 度 24 時間水中保管後 0.9X0.9 ㎜の接着断面ので、引っ張り接着試験を行い、グレープシード抽出物プロアントシアニジンと、クロロヘキシジン群と比較した。接着象牙質の処理効果と、接着試験後の破断面を走査型 電子顕微鏡で観測を行った。さらにナノインデンテーション法により、接着界面の機械的特性評価（弾性率、硬さ評価）を行った。

結果考察：ヘスペリジン配合による、接着象牙質の処理効果に変化はなかった。接着試験の結果、ヘスペリジン配合群は接着強さが有意に上がった。これらはグレープシード抽出物と、クロロヘキシジン群に比べても、有意に高い値であった。また、接着界面の弾性率、硬さ評価ともに、ヘスペリジン配合群は高い値を示した。これらの結果より、ヘスペリジンはプライマー（前処理剤）に配合した場合、その架橋作用により有機物コラーゲンを保護すると考えられ、高いに接着力につながったと思われる。

（３）

方法: 本研究では、コラーゲン保護材としての臨床応用を検討する為、長期耐久性などを評価した。課題(２)の実験系同様に、ヘスペリジン配合（0.5%, 1%, 2%, 5%）セルフエッチングプライマーにて象牙質を前処理し、ヒト象牙質を接着させた。グレープシード抽出物プロアントシアニジンは 0.5%配合した。24 時間水中保管と、長期的接着力の評価のため、1 年間水中保管群を設定した。保管後は、(2) 同様、0.9X0.9 ㎜の接着断面ので、引っ張り接着試験を行い、接着試験後の破断面を走査型 電子顕微鏡で観測を行った。さらに、接着界面の 1 年後の変化を、樹脂含浸層の有機質の劣化に注目し、透過型電子顕微鏡観察評価した。

結果考察：1 年間水中保管群はそれぞれ、接着力が下がったものの、1 年後の接着力を比べると、ヘスペリジン配合しないコントロール群に比べ、

ヘスペリジン 2%と 5%配合のよる群はその低下が少なかった。5%配合群は初期の接着力は 2%より低いが、長期的には接着力を劣化が最も少なかった。透過型電子顕微鏡観察で、ヘスペリジン配合しないコントロール群では、象牙質接着界面の樹脂含侵層中のコラーゲン線維に劣化が見られたが、ヘスペリジン 2%と 5%配合群ではコラーゲンの形態に劣化がみられなかった。コラーゲン線維の架橋効果により、樹脂含侵層中の有機物が保護され、長期的に界面の劣化を抑制したものと考えられる。

結論

フラボノイドは、少量でも抗酸化作用を有し、天然の架橋剤としてコラーゲンを保護すると報告されている。しかし、歯質の再石灰化助長の効果、また修復接着界面下のコラーゲンの補強効果については研究が進んでいなかった。う蝕予防のためには、日常的に安心して使用可能な生体親和性の高い天然フラボノイド(flavonoid)の実用化が大変興味深かった。博士課程の研究で、柑橘由来ヘスペリジン、グレープシード由来のプロアントシアニジンに注目し、歯質象牙質に作用させ、う蝕の進行への影響、接着機能性を評価した。特にヘスペリジンではその効果が確認でき、高濃度では、コラゲナーゼによるコラーゲンの崩壊を抑制し、脱灰抑制、または再石灰化促進効果が見られた。これにより、天然由来の安全性の高い有機化合物であるヘスペリジンの、根面象牙質う蝕治療効果が示唆された。接着処理剤に配合した場合、界面の機械的物性の向上がみられ、短期、長期接着強さが上がった。これは、コラーゲン線維の架橋効果により、樹脂含侵層中の有機物が保護され、長期的に界面の劣化を抑制したものと考えられる。配合の濃度は 2%以上では接着面処理には影響が出る恐れがあるが、長期的コラーゲン保護には、それ以上配合が望ましいと思われる。

Chapter 1

Introduction

The life span of people in Japan as well as around the world is increasing due to the development of modern medicine and public health awareness (Sasada et al., 2009). However, compared to it, the survival rate of natural teeth is not so high especially for the elderly population. The preservation of the natural teeth is an important aspect in maintaining health-related quality of life (Holm-Pedersen et al., 2008; Kimura et al., 2012). Both dental and periodontal healths are essential in improving tooth longevity.

"Super tooth" is a new concept in dentistry. It is based on reinforcement of natural teeth both mechanically and chemically to extend the functional survival. Modified natural teeth are stronger and more stable against dental disease, hence the term "super tooth." In order to achieve it, two big challenges are needed to be overcome. First, caries formation needs to be prevented and the early caries lesion should be reversed. And secondly, the irreversibly damaged tooth structure should be repaired with a durable restoration that would revive the function and aesthetics of the tooth.

Natural products have been used as folk medicine for thousands of years and are promising sources for novel therapeutic agents (Cragg et al., 1997). They have been the focus on by several recent studies as potential materials to prevent oral diseases such as dental caries (Duarte et al., 2003; Nostro et al., 2004; Ooshima et al., 2000). In our consecutive studies, we tried to find the possible role of natural extracts to modify and reinforce natural teeth. Natural extracts such as citrus fruit-derived hesperidin, grape seed-derived proanthocyanidins, green tea-derived Epigallaocatechin Galatea and Genipin are the abundant in various plants and fruits. These natural extracts are gaining research interest in recent years both in preventive and curative medicine. In this thesis paper, we focused on possible ways to use some of these natural extracts for both preventive and curative

strategies against dental caries.

Dental caries is one of the most common oral diseases around the world, especially in under developed countries (Petersen, 2005), which has driven research on new prevention and treatment methods. In order to protect the tooth, the dental hard tissues needs to be modified to resist against caries challenge (Fontana et al., 2009). The balance between de-mineralization and re-mineralization prevent dental caries formation. If the de-mineralization increases due to cariogenic bacteria or food, caries formation takes place. To reverse the caries formation, re-mineralization process need to be promoted to keep the balance. Unlike enamel, dentin is a complex mineralized tissue composed of approximately 70% mineral, 20% organic component and 10% fluid by weight. Type I collagen fiber accounts for 90% of the organic matrix (Ten Cate, 2008). Regulation of dentin caries lesions should take into consideration the interactions of the inorganic carbonated apatite with the collagen matrix. To reverse dentin caries both inorganic apatite as well as the collagen matrix need to be strengthened. In our study we attempted to reinforce both organic and inorganic parts of dentin with natural extracts to prevent and reverse root caries lesions.

The achievement of an efficient and stable bond between tooth substrate and resin materials remains a challenge in restorative dentistry. The primary aim of dental adhesives is to provide retention to composite cements and withstand mechanical forces and shrinkage stress from the lining composite. A good adhesive also should be able to prevent leakage along the restoration's margins. Clinically, failure of restorations occurs more often due to inadequate sealing and hydrolytic degradation of both resin material and dentin collagen that leads to loss of retention (Gaengler et al., 2004; Opdam et al., 2004). The adhesive capacity of

dental adhesives is based on a twofold adhesion. First, the adhesive adheres to enamel and dentin, and second, the adhesive binds to the lining composite. The latter has been shown to be a process of co-polymerization of residual double bonds (–CQC–) in the oxygen inhibited layer. Since dentin is composed of two phases: an inorganic hydroxy-apatite crystal and an organic predominantly type-I collagen matrix, dentin bonding is apparently most effective when the hybrid layer formed between resin monomers and collagen fibrils is structurally stable. Most of the developments in adhesive dentistry have focused on the improvement of bonding agents and technique, while limited investigation has explored the contributions of collagen structure/stability to bond strength. In the second and third chapters we attempted to incorporate natural extracts into a self-etching primer to examine the improvement of dentin bond strength and interfacial mechanical properties of the tooth-restoration complex.

Our studies provide insights into the development of novel strategies for caries prevention, stable dentin bonding and reliable dental adhesive. These strategies can reinforce the natural tooth mechanically and chemically to create "super tooth".

Chapter 2

In *vitro* effect of hesperidin on root dentin collagen and de/re-mineralization

ABSTRACT

The aims of this study were to investigate the effects of hesperidin, a citrus flavonoid, on human root dentin demineralization and collagen preservation, and compare it with chlorhexidine and grape seed extract. Specimens were assigned to different treatment groups: hesperidin, chlorhexidine and grape seed extract. Specimens were subjected to pH cycling by demineralization for 14 h, incubation in testing solutions for 2 h and re-mineralization in presence of bacterial-derived collagenase for 8 h, for 8 days. Calcium release was measured by means of an atomic absorption spectrophotometer, and degraded collagen matrix was investigated by hydroxyproline assay. Specimens were assessed longitudinally with transverse micro-radiography to investigate lesion depth and mineral loss. In hesperidin and grape seed extract groups, demineralization was reduced when the collagen matrix was preserved. The hesperidin group showed the lowest value in lesion depth and mineral loss, indicating that hesperidin inhibited demineralization and probably enhanced re-mineralization even under fluoride-free conditions.

Keywords: Hesperidin, Root dentin, Dentin collagen, Dentin demineralization, Dentin re-mineralization.

1. INTORDUCTION

Root caries is prevalent especially among the elderly population due to gingival recession and exposure of the susceptible root surface (Griffin et al., 2004). During root caries development, two stages are distinguished microscopically. In the first stage, dentin minerals are dissolved by acid produced from bacterial bio-films. In the second stage, the demineralized dentin matrix is further degraded and bacteria infiltrate into the inter-tubular area (AR, 1998). It has been suggested that the presence of an organic matrix may reduce the progression of dentin

erosion (Hara et al., 2003; Vanuspong et al., 2002). Dentin is a complex mineralized tissue composed of approximately 70% mineral, 20% organic component and 10% fluid by weight(Ten Cate, 2008). Type I collagen fiber accounts for 90% of the organic matrix. The preservation and stability of dentin collagen may be essential during the re-mineralization process since it acts as a scaffold for mineral deposition (Bertassoni et al., 2009). Preservation of collagen matrix would be followed by promotion of re-mineralization of demineralized dentin, which is one of the important strategies regarding preventive therapies for root caries (Clarkson and Rafter, 2001; Lynch and Baysan, 2001; Petersson et al., 2007; ten Cate, 2001).

In an attempt to stabilize the collagen matrix, glutaraldehyde and some natural flavonoids such as grape seed extract (GSE) have been used on root caries to investigate their cross-linking effects on demineralized lesion (Hara et al., 2005; Xie et al., 2008). Another approach which has been considered is to inhibit the proteolytic activity of sound and carious dentin (Chaussain-Miller et al., 2006; Sulkala et al., 2001), because the organic matrix of dentin collagen is subjected to degradation by matrix metalloproteinase (MMPs) that present in dentin and saliva (Sulkala et al., 2001). Pharmacological studies have reported that MMPs activity was reduced by the application of chlorhexidine (CHX) which in turn leads to the arrest of carious lesion (Garcia et al., 2009; Kato et al., 2010). Natural products have been used as folk medicine for thousands of years and are promising sources for novel therapeutic agents (Cragg et al., 1997). They have been the focus of several recent studies potential materials in the prevention of oral diseases, particularly plaque-related diseases, such as dental caries (Duarte et al., 2003; Nostro et al., 2004; Ooshima et al., 2000).

A previous study using hesperidin (HPN), a citrus flavonoid, showed that HPN

preserved bovine dentin collagen against proteolytic degradation (Hiraishi et al., 2011). It has also been reported that HPN reduced the susceptibility of dentin lesion to acid dependent demineralization with the potential to promote re-mineralization process (Hiraishi et al., 2011). In this study, we used human root dentin to confirm the effect of HPN to resist collagenous degradation and arrest demineralization. Citrus flavonoids were reported to possess antioxidant (Hirata et al., 2005), anti-inflammatory (Benavente-Garcia and Castillo, 2008), anti-carcinogenic (Miller et al., 2008), hypoglycemic effects (Wood, 2005), and to prevent bone loss (Choi and Kim, 2008; Horcajada et al., 2008). The pH cycling model was employed in the present *in vitro* study to examine the role of collagenase on re-mineralization. Demineralized human root dentin was treated using HPN 5,000 ppm, CHX 2,000 ppm or GSE 5,000 ppm for 2 h a day, which were then subjected to pH cycling for 8 days. The purposes of this study were to investigate the effect of HPN on preservation of collagen matrix, and compare it with that of CHX and GSE. The null hypotheses tested were that HPN, CHX and GSE would (i) not prevent collagen degradation and (ii) not influence de/re-mineralization in human root dentin lesion.

Table 1- Study design. Group PC is positive control and group NC is negative control. DS: demineralizing solution; RS: remineralizing solution. N=6

Group	Demineralization solution for 14 hour	Testing solution For 2 hour	Remineralization Solution for 8 hour
HPN	DS	HPN 0.5%	RS with collagenase
CHX	DS	CHX 0.2%	RS with collagenase
GSE	DS	GSE 0.5%	RS with collagenase
PC	DS	RS	RS with collagenase
NC	DS	RS	RS without collagenase

2. MATERIAL AND METHODS

2.1. Preparation of human root dentin specimens

Thirty non-carious extracted human third molars were used in this study, according to the protocol approved by the Human Research Ethics Committee, Tokyo Medical and Dental University, Japan. Root slabs were obtained from medial and distal root surfaces of human molar teeth and polished to expose the root dentin and to obtain a smooth surface using a series of silicon carbide papers (280, 400, 600, 800, 1000, 1200, 1500, 2000, 4000) up to 4000-grit. The dentin surfaces were then soaked in 10% H_3PO_4 for 10 s to remove the smear layer. One side of each dentin surface was painted with nail varnish, leaving a base line (1st base line) (Figure 4).

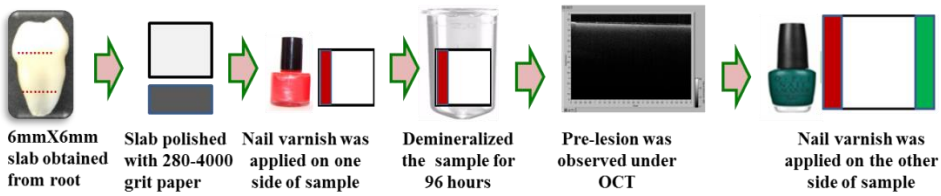

Fig. 1 Specimen preparation

2.2 Lesion formation

After nail varnish application, the specimens were immersed in demineralizing solution (pH=4.5) for 96 h to create pre-lesion. The demineralizing solution contained 50 ml acetic acid, 2.2 mM/L $CaCl_2$ and 2.2 mM/L KH_2PO_4. After pre-lesion formation, the specimens were painted with another coat of nail varnish adjacent to the 1st coat of the exposed dentin surface to create another baseline (2nd base line) (Figure 1). The slabs were divided into five groups (Table1).

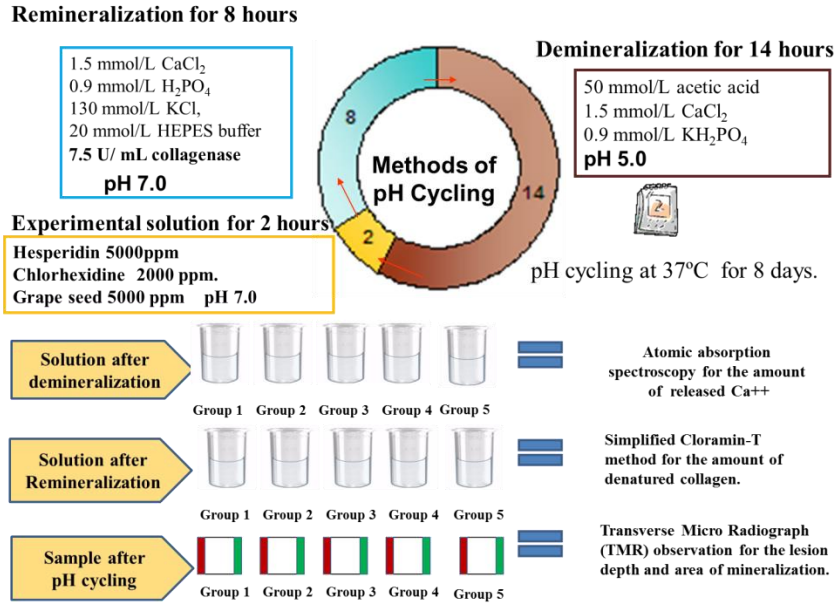

Fig. 2 Method of pH Cycling

2.3. Methods of pH cycling

The demineralizing solution contained 50 mmol/L acetic acid, 1.5 mmol/L $CaCl_2$ and 0.9 mmol/L KH_2PO_4 adjusted to pH 5.0 with KOH. The remineralizing solution contained 1.5 mmol/L $CaCl_2$, 0.9 mmol/L KH_2PO_4, 130 mmol/L KCl and 20 mmol/L HEPES buffer, adjusted to pH 7.0 with KOH. Highly purified collagenase type VII from Clostridium histolyticum (C-0773, Sigma Chemical Co., St. Louis, MO, USA) was further added to obtain a re-mineralization solution that contains 7.5 U/mL collagenase. The testing solution was prepared by adding HPN (hesperidin, Wako Pure Chemical Industries, Ltd., Tokyo, Japan), CHX (chlorhexidine di-gluconate, Sigma–Aldrich, St. Louis, MO, USA) or GSE (Gravinol® grape seed extract, Kikkoman, Chiba, Japan) to the re-mineralization solution. The concentrations used for HPN, CHX and GSE were 5,000 ppm, 2,000 ppm and 5,000 ppm, respectively. Each specimen was immersed individually at 37ºC in the demineralizing solution for 14 h, in the testing solution

with HPN, CHX or GSE for 2 h and in the re-mineralizing solution for 8 h. The specimens were thoroughly rinsed between each immersion with buffer solution containing 130 mmol/L KCl, 20 mmol/L HEPES, adjusted to pH 7.0 with KOH. The pH cycling was performed at 37ºC for 8 days.

2.4. Chemical analyses

2.4.1. Determination of calcium ion release

The demineralizing solution was collected to measure the amount of calcium ion dissolved during the pH cycling. An aliquot of 2.9 ml of 1.67% $LaCl_3$ $7H_2O$ in 50 mM HCl was added to each 100 ml of collected solution. The atomic absorption was measured using an atomic absorption spectrophotometer (AA-6300, SHIMADZU.INC, Kyoto, Japan) at 423 nm. The solution after the first demineralization per each sample was used as an individual baseline of calcium release. Then, the total calcium ion release from the 1st day to the 8th day during the pH cycling was measured and expressed in relative release compared with that of the baseline.

2.4.2. Determination of degraded collagen

Degradation of collagen was determined by estimating hydroxyproline, an amino acid characteristic of collagen. The total re-mineralizing solution was collected after the pH cycling. Sixty micro-liters of the solution (from stock solution collected in each day) were subjected to chemical analysis of degraded collagen using the simplified chloramines-T method (Reddy and Enwemeka, 1996).In brief, the solution aliquot was hydrolyzed with 2N sodium hydroxide by autoclaving at 120°C for 20 min. The cloramines-T was added to the hydrolyzate to allow oxidation, followed by the addition of Ehrlich's aldehyde reagent for hydroxyproline assay. When chromophore was developed, the absorbance of each specimen was read at 550 nm using a spectrophotometer (BIO-RAD-680 Micro

plate reader, BIO-RAD laboratories, Tokyo, Japan) and converted to concentration of hydroxyproline. Amount of hydroxyproline was determined by plotting the value with standard calibration curve.

2.5. Transverse micro radiography (TMR) measurements

After the pH cycling for 8 days, the mineral loss (ΔZ, vol% mm) and lesion depth (LD, μm) were examined using TMR. The specimens were sectioned longitudinally through the lesion centre into 220±20 μm thicknesses. The cut sections were put in a solution containing 80% of glycerin and 20% of water to prevent shrinkage. Photo plates (HY2, Konica Minolta Holdings, Inc., Tokyo, Japan) were exposed at 4 mA and 25 kV for 5min using an X-ray generator (Type SRO-M50, Sofron Company Lit., Tokyo, Japan) together with 15 sheets of an aluminum Al step wedges for calibration. The plates were developed and fixed according to standard techniques. The microradiographs were analyzed under a microscope (BX 41, Olympus Co., Tokyo, Japan) with CCD camera (DP70, Olympus Co., Tokyo, Japan). The LD was defined as the distance from the surface of the second baseline to the lesion where the mineral content was more than 95% of the sound dentin. The ΔZ was determined by plotting the vol% mineral profile towards the LD in each specimen section with the sound dentin set as 48 vol% mineral contents (Inaba et al., 1997).

2.6. Statistical analysis

The effect of testing solution on the calcium release, degraded collagen and TMR variables (ΔZ and LD) was tested by one-way ANOVA using statistical software package (SigmaStat Version 16.0, SPSS, Chicago, IL, USA). Where appropriate, post-hoc Tukey multiple comparisons tests were performed on all groups. The level of statistical significance was set at 5%.

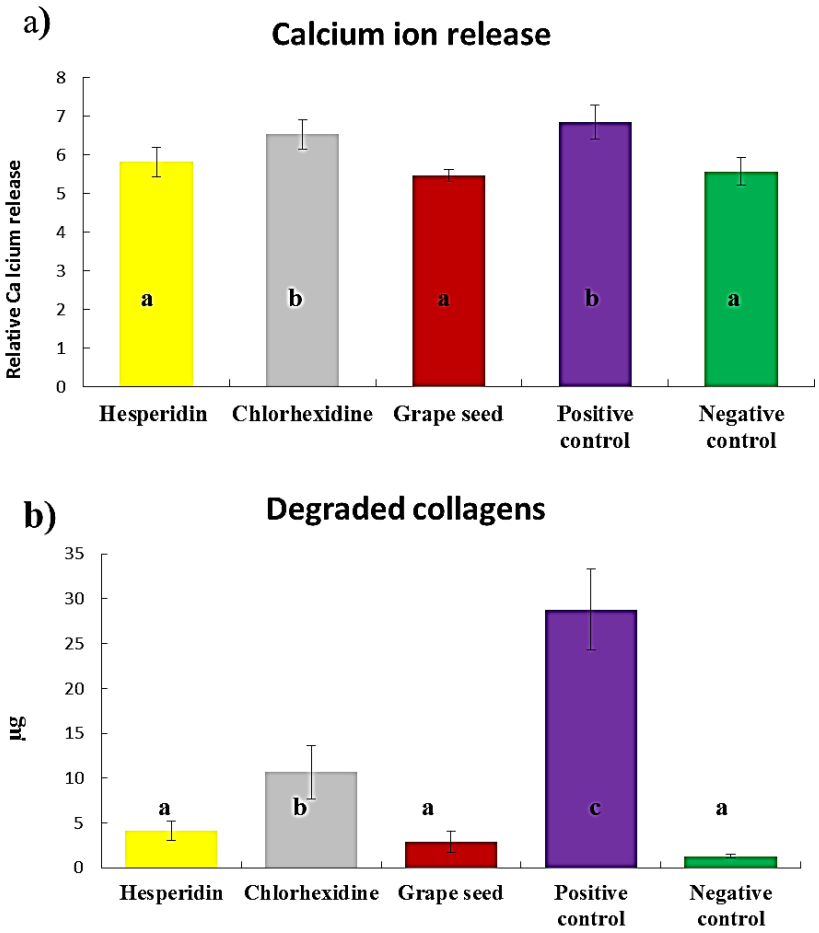

Fig. 3 (a) Calcium release during demineralization in pH cycling and (b) hydroxyproline detection (µg per sample) during re-mineralization in pH cycling (mean and SD). Note: (i) Calcium release was expressed in relative release as the amount of calcium of total release for 8 days compared to that of the first day demineralization, and (ii) Same letters indicate no significant difference at $p>0.05$.

3. RESULTS

The results of chemical analyses are shown in Figure 3 (a, b). The one-way ANOVA indicated that the effect of testing solutions had a significant difference

on the results of chemical analyses ($p<0.05$ for calcium release; $p<0.01$ for degraded collagen). A significant difference was revealed in the results between the negative and positive controls ($p<0.001$ for both calcium release and degraded collagen), indicating that the incubation with the presence of collagenase had an effect of causing mineral dissolution and organic degradation. Regarding calcium release results, the lowest amount was for GSE group, followed HPN and CHX groups, respectively. According to the results of degraded collagen, HPN and GSE groups showed the lowest value with no statistical significant difference when compared with the negative control ($p>0.05$). The results of TMR measurement are shown in Figure 4 (a, b). The effect of testing solutions had a significant difference on the TMR variables ($p<0.01$ for LD and $p<0.001$ for ΔZ). No significant difference in LD was shown between the positive control and CHX group ($p>0.05$). The positive control showed a statistical significant ΔZ when compared with HPN and GSE groups. Representative TMR images are shown in Figure 5 (a-e). The image manifested the effect of HPN on resisting demineralization (Figure 8a).

4. DISCUSSION

The chemical analyses demonstrated that HPN, CHX and GSE preserved and stabilized dentin collagen. The calcium release in HPN and GSE groups was significantly different when compared with the positive control, while no significant difference was found between the CHX group and the positive control. Thus, the first null hypothesis was partially rejected. The positive and negative controls were significantly different in TMR measurement. The results of TMR revealed that the LD and ΔZ were lower in groups incubated with HPN and GSE, when compared with the positive control. This result implies that HPN and GSE had the effect to prevent demineralization and/or promote re-mineralization. Thus,

the second null hypothesis was partially accepted.

In a previous study, it was reported that demineralization of the surface was observed on dentin samples when the pH cycling was performed with collagenase even at pH 7.0 in the re-mineralizing solution (Kawasaki and Featherstone, 1997). The authors claimed that surface demineralization might occur when mineral contents released as the result of degradation of collagen matrix.

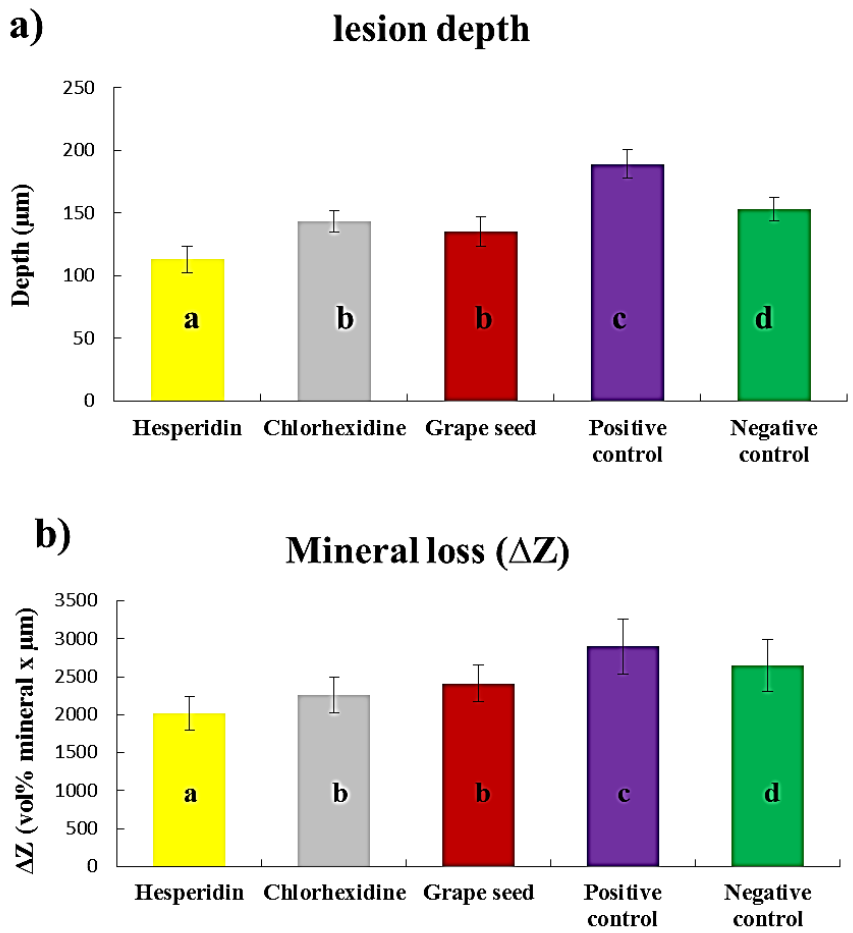

Fig. 4 Lesion depth (LD) (a) and mineral loss (ΔZ) (b) after pH cycling (mean and SD). Note: same letters indicate no significant difference at $p>0.05$.

Likewise; in our present study, the mineral release might have occurred during re-mineralizing cycles, which accounted for increased ΔZ for the positive control (using collagenase in the re-mineralizing solution). Such a mineral release was suppressed for the negative control group (without using collagenase) and those groups using HPN, CHX and GSE where collagen matrix was preserved by the aforementioned agents. Although the amount of mineral release in re-mineralizing cycles was not chemically analyzed, the results of TMR indicated the effect of stabilized collagen matrix on mineral content in the lesion.

Dentin organic matrix plays an important role in demineralization and re-mineralization process. The organic layers of dentin are important in hampering the lesion progression and preventing further demineralization challenges (Hara et al., 2005). In previous studies using collagenase, the proteolytic degradation of demineralized matrix was said to enhance the susceptibility of dentin lesions to acid-dependent demineralization (Fukuda et al., 2009; Walter et al., 2008). Recent studies have demonstrated that CHX possesses a potent anti-proteolytic effect due to its ability to inhibit activity of MMPs in carious dentin (Garcia et al., 2009).The present study demonstrated a positive effect of CHX in preserving dentin collagen against bacterial proteolysis, which contributed to the low value of organic matrix degradation. However, the TMR measurement showed that the incubation in CHX did not contribute to suppressed ΔZ. This finding was consistent with that of a previous study involving bovine root dentin (Hiraishi et al., 2011). The preserved organic matrix might hamper further diffusion of calcium and phosphate ions out of the dentinal lesion of caries, resisting further demineralization.

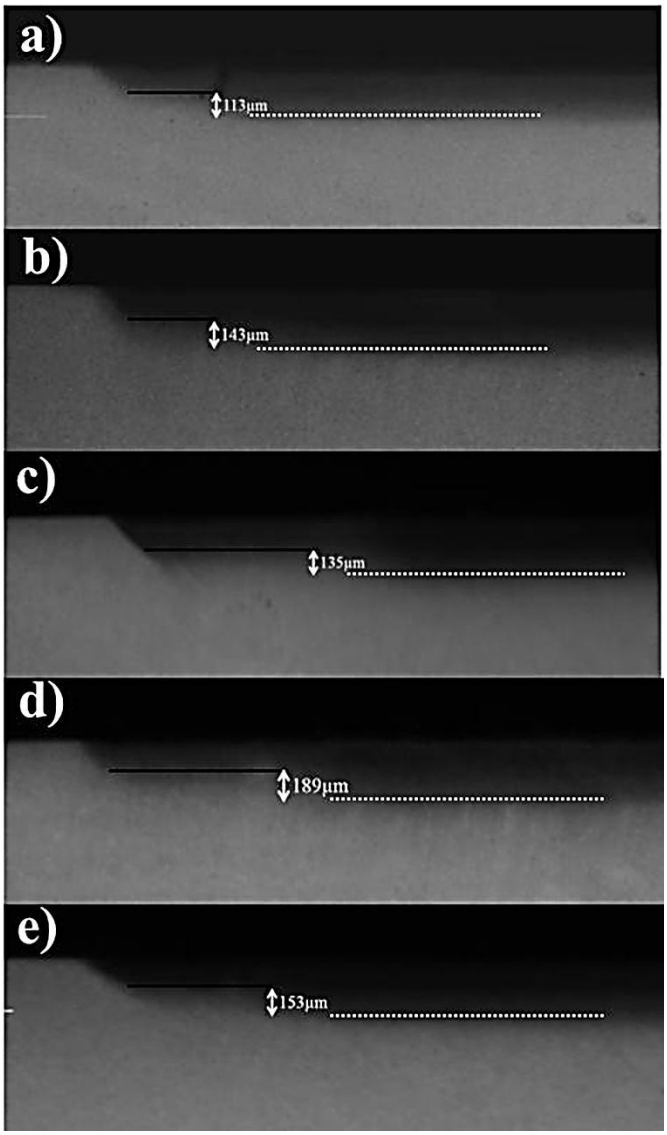

Fig.5 Representative transverse micro-radiographic images, actual lesion depth and mineral loss was measured from the baseline (black line in the image) to the lesion where the mineral content was more than 95% of the sound dentin (white line in the image) (a) HPN (LD, 113µm; ΔZ, 2015vol% µm); (b) CHX (LD, 143µm; ΔZ, 2259vol% µm). (c) GSE (LD, 135µm; ΔZ, 2413vol% µm); Positive control (LD, 189µm; ΔZ, 2899vol% µm); (e) Negative control (LD, 153µm; ΔZ, 2654 vol% µm).

In the HPN and GSE groups, the effect of incubation in HPN and GSE was revealed in terms of preserved collagen and reduced LD and ΔZ. With the limited findings under TMR analysis, the difference in re-mineralization process between the flavonoid groups (HPN and GSE) and CHX group could not be thoroughly explained. Further studies should be conducted to investigate the chemical reactions of HPN and GSE with calcium and/or phosphate ion. The suppressed ΔZ in the HPN group may be explained by the contribution of HPN on stabilization of the exposed collagen matrix. Xie et al reported that GSE is a promising agent to be used in non-invasive root caries therapy as re-mineralization was evident on root dentin most likely through the interaction between GSE and proteins in dentin thus stabilization of dentin matrix (Xie et al., 2008). Previous studies using glutaraldehyde demonstrated that fixing the collagenous/non-collagenous protein and cross-linking collagen matrix reduced the progression of the root caries development (Hara et al., 2005; Walter et al., 2008). The stabilized collagen matrix behaves as a mechanical barrier to mineral diffusion (Dijkman et al., 1992), thereby resisting demineralization and promoting re-mineralization. The preservation and stability of dentin collagen may be essential during the re-mineralization process since it acts as a scaffold for mineral deposition (Bertassoni et al., 2009). Furthermore, non-collagenous proteins in dentin that possess the mineral-inductive capacity have a crucial function on the organic matrix for apatite precipitation (Lussi and Linde, 1993). In our speculation, the effect of HPN on re-mineralization may be related to its interaction with collagen and/or non-collagenous proteins, resulting in stabilizing collagen matrix and induction of re-mineralization. In fact, the binding capacity of HPN to human serum albumin protein indicated that HPN played a comprehensive role in homeostatic process (Liu et al., 2004).

5. CONCLUSIONS

Within the limits of this *in vitro* study, the HPN may have the potential to promote re-mineralization process. The biochemical mechanism of HPN has not been investigated and its application in dentistry has not been developed. Further studies are needed to elucidate the mode of action of HPN on human dental tissue.

Chapter 3

Effect of natural cross-linkers incorporation in a self-etching primer on dentine bond strength

ABSTRACT

Objectives: The aim of this in-vitro study was to investigate the effect of incorporation of natural cross-linkers into the primer of a self-etching adhesive on resin-dentine bond strength.

Methods: Flat dentine surfaces were prepared from extracted human molar teeth and were applied with the following self-etching primers. The 0.5% hesperidin (HPN), 0.5% chlorhexidine (CHX) or 0.5% grape seed extract (GSE) was incorporated into Clearfil SE primer (Kuraray Medical Inc.) to formulate three experimental primers. The original SE primer served as control. Following primer application, the teeth were bonded with Clearfil SE bond, restored with resin composite and stored in water for 24 hours at 37 ºC. The bonded specimens were sectioned into beams and subjected to micro tensile bond testing (μTBS). Failure analysis and morphological evaluation of the dentine surfaces were performed using a scanning electron microscope (SEM). Hardness (H) and elastic modulus (EM) were measured using nano-indentation technique to examine the mechanical properties of the bonded interfaces. Results: One-way ANOVA showed significant differences in μTBS, H and EM among the tested and control groups ($p<0.001$). Tukey post-hoc test revealed that incorporation of HPN significantly increased μTBS, H and EM, when compared with the other groups ($p<0.006$). The GSE-incorporated group significantly decreased μTBS, H and EM, when compared with the other groups ($p<0.006$); while CHX- incorporated group did not show any statistical significant difference when compared with the control group.

Conclusion: Incorporation of HPN into Clearfil SE primer had a positive influence on the immediate μTBS and mechanical properties of the bonded interface.

Key words: Dentine bond strength; Hybrid layer; Cross-linker; Nano-indentation; Self-etching adhesive.

1. Introduction

Despite recent improvements in dental adhesive systems, the bonded interface remains the weakest area of the tooth-colored restorations. Degradation of the hybrid layer adversely affects the resin-dentine bonds, which can eventually lead to failure of the adhesive restorations (Breschi et al., 2008). This degradation, caused by both hydrolytic degradation of resin and collagen fibrils (Hashimoto et al., 2003), would leave some collagen fibrils unprotected and vulnerable to the action of host-derived dentinal matrix metalloproteinase (MMPs) enzymes (Carrilho et al., 2007a; Pashley et al., 2004). The detrimental role of MMPs in dentine bonding has been documented (Zhang and Kern, 2009). The intrinsic MMPs in dentine can be activated by the acidic properties of adhesive systems. Mild acids are known to activate endogenous MMPs in dentine (Nishitani et al., 2006b; Tay et al., 2006). Both etch-and-rinse (Mazzoni et al., 2006) and self-etch adhesives(Nishitani et al., 2006b) have the ability to reactivate gelatinases (MMP-2 and MMP-9) and collagenase in de-mineralized dentine powder(Mazzoni et al., 2007). Self-etch adhesives may activate latent MMPs to near-maximum levels, causing degradation of resin-dentine bonds (Nishitani et al., 2006b).

Several approaches have been suggested to resist collagen degradation and to preserve resin-dentine bond strength. The use of MMP inhibitors either before the application of the adhesive (Carrilho et al., 2007b) or as a component of the adhesive (Zhou et al., 2009) is a promising approach. Chlorhexidine (CHX), a potent non-specific MMP-inhibitor (Gendron et al., 1999), has been reported to arrest degradation of the hybrid layer (Hebling et al., 2005). Another approach to

resist collagen degradation is the use of cross-linking agents. Cross-linkers such as tannic acid have been found not only to lower the rate of enzymatic degradation of collagen, but also to increase the mechanical properties of dentine (Bedran-Russo et al., 2009). Other cross-linkers that have been used with the result of increased collagen stability and bond strength are glutaraldehyde (GA) and grape seed extract (GSE) (Al-Ammar et al., 2009; Macedo et al., 2009). Epigallaocatechin gallate, the most abundant catechin in green tea (Wang et al., 2008), has also been shown to reduce dentine erosive demineralization, possibly through its MMPs-inhibition property (Magalhaes et al., 2009).

The flavonoids are polyphenolic compounds that are found in foods of plant origin. Over 4000 structurally unique flavonoids have been recognized (Guardia et al., 2001). Hesperidin (HPN), hesperetin-7-O-rutinoside, is a flavonoid extracted from citrus fruits. The medical benefits of HPN include antioxidant, anti-inflammatory and anti-carcinogenic effects. It has also been reported that HPN influences bone formation (Trzeciakiewicz et al., 2010). We first attempted the use of HPN on dental tissue using in vitro caries model to demonstrate that the cross-linking properties of HPN may resist collagenous degradation and arrest demineralization of bovine root dentine (Hiraishi et al., 2011). In order to consider the cross-linking effect of HPN on dentine collagen, the present study was attempted to examine the effect of HPN on dentine bond strength of a self-etch adhesive. Thus the objectives of this study were to evaluate the effect of HPN incorporation into a self-etching primer on dentine bond strength and mechanical properties of the bonded interface, and compare with the effect of CHX and GSE. The mechanical properties were evaluated using the nano-indentation method, which measured the hardness (H) and elastic modulus (EM) of the dentine adhesives and resin-dentine interface. The null hypotheses

tested in this study were (i) incorporation of HPN, CHX or GSE has no effect on the immediate bond strength to dentine and (ii) incorporation of these agents has no effect on the mechanical properties of resin-dentine interface.

Group	Materials tested	pH
1	Clearfil SE primer	2.0
2	Clearfil SE primer + 0.5% Hesperidin	2.0
3	Clearfil SE primer + 0.5% Chlorhexidine	2.0
4	Clearfil SE primer + 0.5% Grape seed Extract	2.0

Clearfil SE primer	**Clearfil SE bond**
•MDP	•MDP
•HEMA	•HEMA
•Dimethacrylate monomer	•Dimethacrylate monomer
•Water	•Micro filler
•Catalyst	•Catalyst

Table.2 Study design and composition of SE primer and SE bond

2. Materials and methods

2.1. Tooth preparation

The teeth used in this study were collected after obtaining the patients' informed consent. The Human Research Ethics Committee of Tokyo Medical and Dental University, Japan reviewed and approved this study under protocol number 725. Sixteen freshly extracted non-carious human molar teeth were used for bond strength testing. A flat dentine surface was created perpendicular to the tooth's longitudinal axis using a slow-speed diamond saw (Isomet, Buehler Ltd., Lake Bluff, IL, USA) under water cooling to remove occlusal dentine. Smear layer was produced on each surface using #600 SiC paper under water irrigation. Four teeth for each group were allocated for the following self-etching primers. HPN (hesperetin-7-O-rutinoside, Wako Pure Chemical Industries, Ltd, Tokyo, Japan), CHX (Chlorhexidine di-acetate, Sigma-Aldrich, Saint Louis, MO, USA) or GSE

(proanthocyanidins, Kikkoman Biochemifa, Chiba, Japan) was added to Clearfil SE primer (Kuraray Medical Inc. Tokyo, Japan) to formulate the experimental primer groups (Table 1). The concentration was 0.5 wt % for all groups. The pH value of each experimental primer was measured with a digital pH meter (Twin pH B-211, HORIBA, Ltd. Kyoto, Japan). The original SE primer served as control. The dentine surfaces were conditioned with the primers according to the manufacturer's instructions, then Clearfil SE bond (Kuraray Medical Inc. Tokyo, Japan) was applied and light-cured for 10 seconds (OPTILUX 501, Kerr corporation, CA, USA. light intensity 650mW/cm^2). Composite resin (Clearfil AP-X, Kuraray Medical Inc. Tokyo, Japan) was placed on the dentine surfaces incrementally up to 5 mm of thickness. Each increment was light-cured for 30 seconds.

2.2. Micro tensile bond strength testing

After storage in de-ionized water at 37ºC for 24 hours, the bonded teeth were sectioned longitudinally into serial slabs, and further sectioned to obtain (0.9mm x 0.9mm) composite-dentine beams. The exact dimension of each beam was measured using a pair of digital calipers. Any specimen close to pulp horns was discarded due to inadequate size of dentine for tensile testing. The number of beams used for the bond testing ranged from 12 to 16 from one tooth. Each beam was attached to the test apparatus with an adhesive (MODEL REPAIR II BLUE, DENSPLY-Sankin. Ohtawara, Japan) and stressed to failure under tension using a universal testing machine (EZ Test, Shimadzu Co. Kyoto, Japan) at a crosshead speed of 1 mm/minute.

2.3. Failure mode analysis

The fractured dentine surface was air-dried, sputter-coated with gold/palladium and examined using a scanning electron microscope (SEM, JSM-5310LV

scanning microscope, JEOL Ltd. Tokyo, Japan) operating at 5 kV. The failure modes were categorized into four groups according to the type and location, (A) mixed type of failure in resin composite and adhesive layer; (B) cohesive failure in adhesive layer; (C) mixed type of adhesive failure at the interface with retention of adhesive; (D) cohesive failure in dentine.

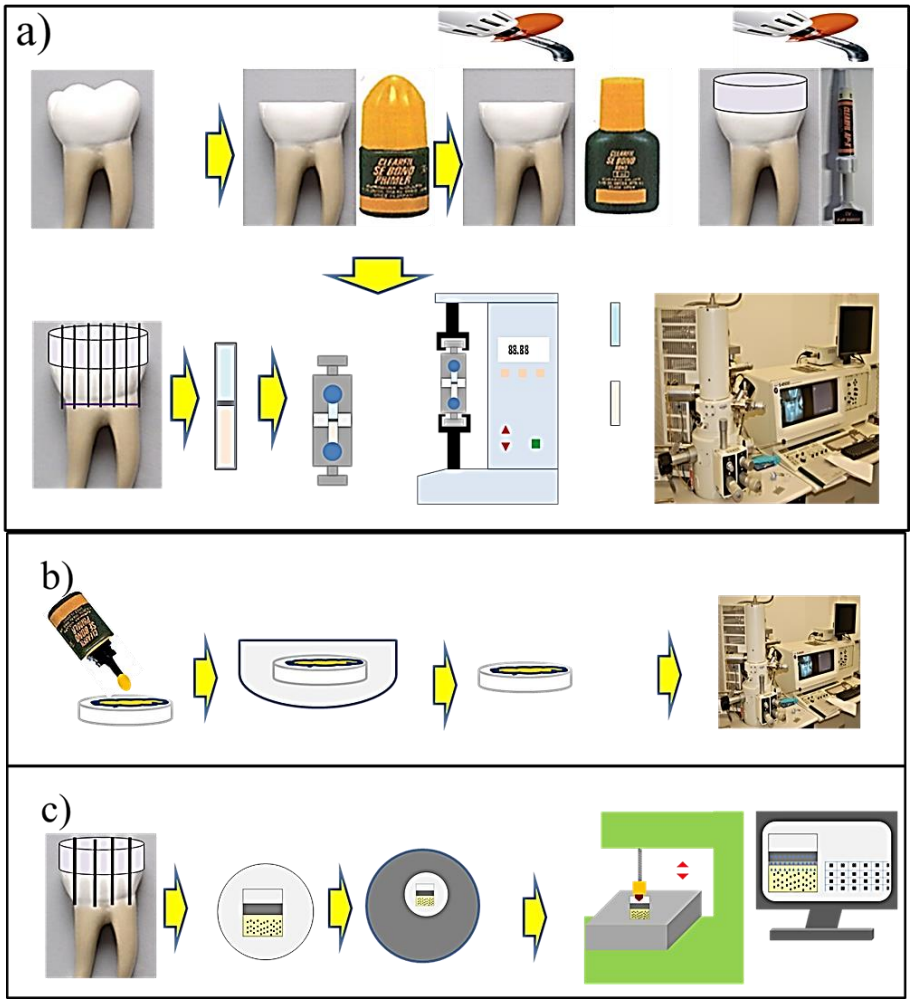

Fig. 6 Schema of a) Bond strength and failure mode, b) Surface morphology, c) Mechanical properties

2.4. Nano-indentation tests

Nano-indentation tests were conducted to measure H and EM of the adhesive layers and bonded interfaces. Dentine surfaces were prepared from 12 freshly extracted teeth. Three teeth in each group were bonded as previously described for tooth preparation. After storage in de-ionized water for 24h at 37°C, the specimens were sectioned perpendicular to the bonding surface around 2mm thick. Three slabs of 2 mm thickness were selected from the center of each bonded specimen. The specimens were embedded in epoxy resin with the resin–dentine interfaces facing out, polished under running water on a series of SiC paper with grits ascending from #600 to #2000 and finally polished with diamond pastes of decreasing particle sizes down to 0.25 μm. After polishing, nano-indentation test was performed at a constant temperature of 27.5°C with a Berkovich indenter attached to a computer-controlled nano-indentation device (ENT-1100, Elionix. Tokyo, Japan). The positions of indentation points were programmed at an interface, 10μm distance from the interface in the adhesive layer and in the dentine. Data of H were assigned to H_1 (hardness at the adhesive layer), H_2 (hardness at the interface) and H_3 (hardness at dentine). The relative hardness at the interface ($H_{2/3}$) was calculated as the ratio of H_2 to H_3 (i.e. $H_{2/3}=H_2/H_3$) to consider intrinsic difference in hardness of individual dentine substrate. The data of EM were determined in the same manner and assigned to EM_1, EM_2, EM_3 and $EM_{2/3}$. The indents were observed with a CCD camera at 20X magnification connected to the device to exclude irregular or unclear shaped indentations. After a series of pilot indentations were performed to select appropriate loading regime, data were obtained from 30 successful indentations (10 points in each adhesive/ interface/ dentine) on each sample made at a constant loading rate of 10 gf/s with the load increasing until a maximum value of 300 mgf (approximately 3.0 mN).

The constant rate was maintained by adding a load increment of 0.1 mgf to the current load per 10 ms interval. After this step-loading segment, the maximum load was held for 1 second, followed by the unloading segment in which the load was gradually removed. The hardness was calculated as follows:

Hardness = Load/Ap, where Ap is the projected area of the indentation at the maximum load, which depends on the indenter geometry and the penetration depth calculated according to the index provided by the device manufacturer.

The elastic modulus was determined as follows:

Elastic modulus= (dP/dh) ($\sqrt{\pi}/2\sqrt{A}$) where dP/dh is the slope of the unloading load–displacement curve at the maximum load, and A is the contact area created by the indentation.

The data were obtained from the average value of 10 points of each location. Statistical analysis was performed for 9 specimens (3 slabs each from 3 bonded teeth) in each group.

2.5. Examination of the self-etching effect of experimental primers on smear layer

To examine the effect of experimental SE primers on the smear layer, dentine discs of approximately 1 mm thickness were obtained from the mid-coronal dentine of another four extracted human third molars. The dentine surfaces were similarly treated with the pure primer or each of the experimental primers for 20 seconds. Immediately after treatment, the discs were soaked in 100% acetone for 5 minutes to remove the applied primer. They were then dehydrated in ascending concentrations of ethanol, followed by immersion in hexamethyldisilazane (Wako Pure Chemical Industries, Ltd, Tokyo, Japan) for 10 minutes and mounted on aluminum stubs and sputter-coated with gold/palladium and examined with the SEM operating at 5 kV.

2.6. .Statistical analysis.

The effects of experimental primers on μTBS, H and EM data were tested by one-way ANOVA using a statistical software package (Sigma Stat Version 16.0, SPSS. Chicago, IL, USA). In case of significant, further statistical analyses were performed by Tukey multiple comparisons test. The level of statistical significance was set at 5%.

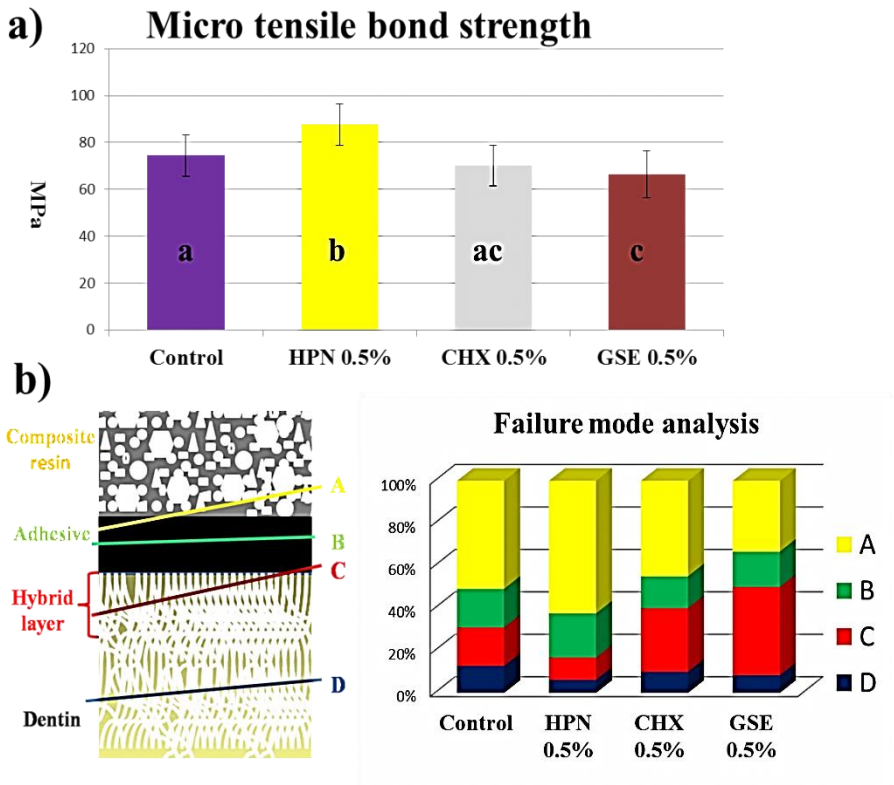

Fig. 7 a) Micro tensile bond strength after 24h water storage, Control group (74.4±8.8MPa: n=55), HPN-incorporated group (87.7±8.9 MPa: n=48), CHX-incorporated group (70.1±8.7 MPa: n=40) and GSE-incorporated group (66.5±13.8 MPa: n=45). b) Location and failure percentage of dentine side of fractured specimens during bond strength measurement.

3. Results

3.1 Micro tensile bond strength

Fig. 7a shows the μTBS of the experimental and control groups. One-way ANOVA showed significant differences among the tested groups ($p<0.001$). Tukey post-hoc test revealed that the μTBS of HPN-incorporated group (87.7 ± 8.9 MPa: n=48) was significantly higher, when compared with the control group (74.4 ± 8.8 MPa: n=55) and experimental groups ($p<0.006$). Although the CHX-incorporated group (70.1 ± 8.7 MPa: n=40) showed lower bond strength, when compared to the control group, it was not statistically significant ($p>0.05$). The GSE-incorporated group (66.5 ± 13.8 MPa: n=45) showed significantly lower bond strength when compared to the control group ($p<0.001$).

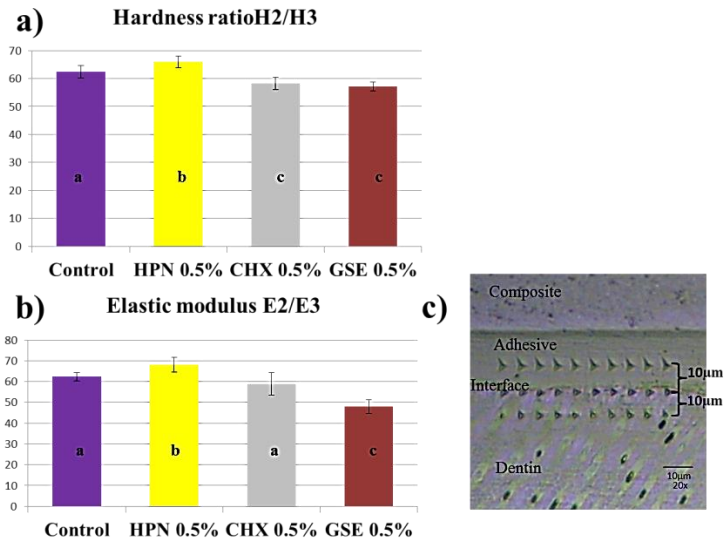

Fig. 8 Mechanical properties of resin-dentine interface. a) Hardness ratio (H2/H3): Control group (62.4±2.2), HPN-incorporated group (65.9±2.1), CHX-incorporated group (58.3±2.2) and GSE-incorporated group (57.2±1.5). b) Elastic modulus ratio (E2/E3): Control group (62.3±2), HPN-incorporated group (68.1±3.5), CHX-incorporated group (58.8±5.3) and GSE-incorporated group (48.0±3.2).

3.2 Failure mode analysis

Fig. 7b shows the fracture patterns and percentage of failure modes of the dentine sides. Most of the observed failures in the control group were cohesive failure in dentine and adhesive. Percentage of type C failure, which involved adhesive failure at the bonded interface, decreased with higher μTBS in HPN-incorporated group and increased with lower μTBS in CHX-incorporated, and GSE-incorporated groups. Representative SEM images from each group are shown in Fig. 9.

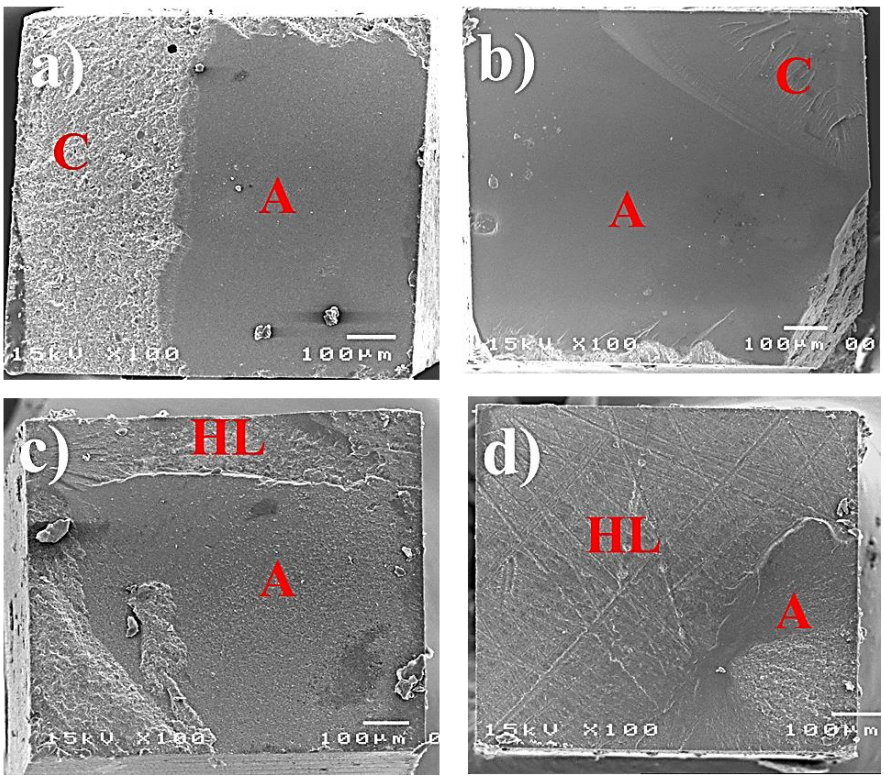

Fig. 9 Representative SEM images of fractured dentine surfaces: a) control group; b) HPN incorporated group; c) CHX incorporated group; d) GSE incorporated group. (C) Represents composite, (A) represents adhesive and (HL) represent hybrid layer.

3.3 Nano-indentation tests

One-way ANOVA showed significant differences among the tested and control groups ($p<0.001$). Tukey post-hoc test revealed that the hardness ($H_{2/3}$) and elastic modulus ($EM_{2/3}$) ratio of HPN-incorporated group were significantly higher, when compared with the control and experimental groups. The GSE-incorporated group showed the lowest $H_{2/3}$ and $EM_{2/3}$ ratio among the entire tested groups (Fig. 8). The hardness and elastic modulus of adhesive (H1, E1) did not show any statistically significant difference among the tested groups (data not shown).

3.4 Self-etching effect of experimental primers on smear layer (Figure 10)

SEM micrographs showing the self-etching effect of the experimental primers on smear layer are shown in Fig 4. Observation of dentine surfaces etched with the experimental primers did not show any remarkable differences among the groups, except for some smear-plug occluded dentinal tubules that were observed in the GSE-incorporated group (Fig. 10d).

4. Discussion

The effect of natural cross-linkers incorporation into a self-etching primer on the immediate bond strength and the mechanical properties of the bonded interface were addressed in our study. The results showed that the incorporation of HPN increased the immediate bond strength to dentine, while GSE significantly reduced this bond strength. The incorporation of CHX did not affect the bond strength. The incorporation of HPN improved the H and EM of the resin-dentine interface; however, such a positive effect was not revealed for CHX and GSE. Thus, the null hypotheses were rejected.

Our previous study using bovine dentine specimens revealed that the treatment with HPN improved resistance of dentin collagen against enzymatic degradation (Hiraishi et al., 2011). In the present study, the incorporation of HPN improved

the H and EM of resin-dentine interface. Since Miguezl *et al* reported that mechanical properties of collagen matrix significantly contribute to the tensile strength of dentine (Miguez et al., 2004), we speculate that HPN may stabilize collagen matrix and improve mechanical properties of the hybrid layer, which consequently produced the high bond strength.

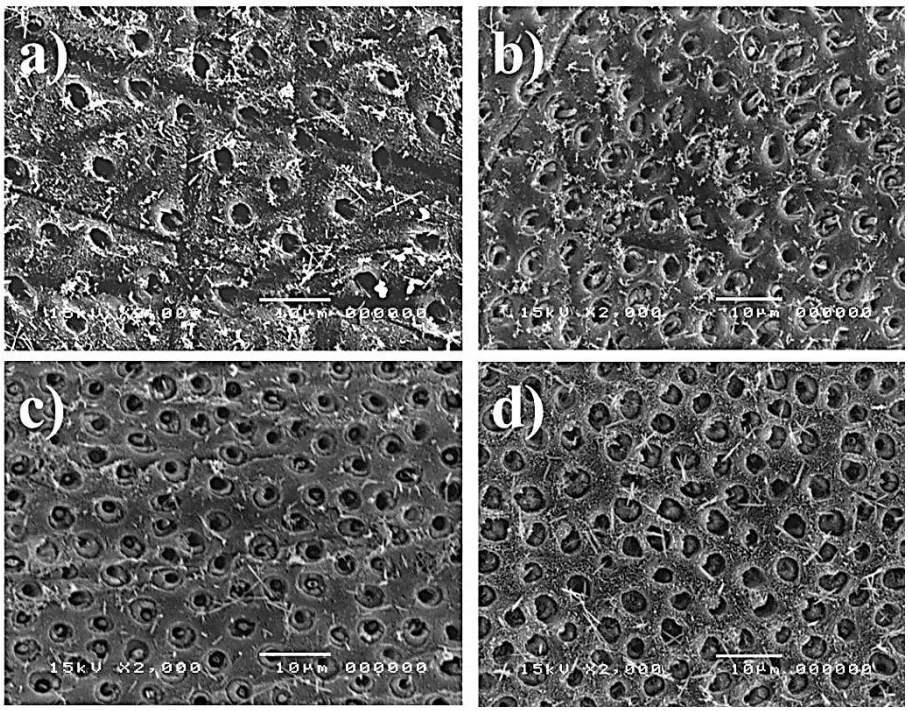

Fig.10 SEM images of dentine surface after applying each experimental primer: a) Original SE primer; b) HPN-incorporated primer; c) CHX-incorporated primer; d) GSE-incorporated primer.

In an attempt to stabilize collagen matrix, GA and natural cross-linkers, such as GSE have been used in human dentine to demonstrate their effects on demineralized lesion (Bedran-Russo et al., 2007; Walter et al., 2008). GSE composed mainly of oligomeric proanthocyanidins, which is well known as a natural collagen cross-linker. The cross-linking effect of proanthocyanidins is

attributed to its interaction with proline-rich proteins such as collagen (Frazier et al., 2010). This compound was shown to increase the mechanical properties of dentine through its cross-linking action (Braga et al., 2000). Although the cross-linking effect of HPN has not been well elucidated, the fundamental structural unit in HPN is similar to that of grape seeds proanthocyanidins (Haslam, 1996), Both HPN and proanthocyanidins are phenolic flavonoids, possessing a chroman ring; therefore, the chemical action of HPN on collagen might be explained in the same manner as the cross-linking effect of proanthocyanidins.

Several studies have investigated the effect of GSE on dentine bond strength when it was applied on etched dentine prior to bonding with etch-and-rinse adhesive systems, reporting that pre-treatment with GSE improved the bond strength (Al-Ammar et al., 2009; dos Santos et al., 2011; Green et al., 2010; Macedo et al., 2009). They postulated that the increase in mechanical properties of GSE-treated dentine is the cause of improved bond strength of the etch-and-rinse adhesives. On the other hand, we used a self-etching adhesive system to attempt the first trial to incorporate GSE into a self-etching primer. Regarding its effect on bond strength, our results are inconsistent with those obtained by utilizing etch-and-rinse systems. Possible explanation for the different results is the use of different adhesive systems. An interesting observation in our study was the occlusion of dentinal tubules by smear particles in dentine conditioned with GSE-incorporated primer. This poorly dissolved smear layer could be the cause of low bond strength values obtained in this group. This adverse effect of GSE on dissolving smear lays is related to the apparent molecular size of GSE. Since GSE contained oligomeric proanthocyanidins, the apparent molecular size might be manifold. The chemical with increased molecular size would compromize the penetration and diffusion of functional

monomers of the primer. The amount of monomer-diffusion into the demineralized dentine and polymerization of diffused monomers account for the quality of the bonding systems (Nakabayashi et al., 1982). Incomplete infiltration of resin monomer may hamper ideal hybridization of dentine and monomers, leading to low bond strength.

The CHX group was designed to compare the effect of GSE and HPN when incorporated in the primer because the addition of CHX in self-etching primers has been attempted in previous studies (de Castro et al., 2003; Ricci et al., 2010; Stanislawczuk et al., 2011). In our study, the incorporation of CHX in the self-etching primer did not show any significant differences in the immediate bond and mechanical properties of resin dentine interface, when compared with the control group. The application of CHX, known to have a MMP-inhibitory effect through its zinc cation-chelating property (Osorio et al., 2011), significantly improved the immediate bond strength of both etch-and-rinse adhesive and self-etch adhesive (de Castro et al., 2003; Ricci et al., 2010; Stanislawczuk et al., 2011). More importantly, the long-term bond durability was improved after 12 months, when CHX was incorporated into a self-etching primer or applied on etched dentine prior to bonding (Zhou et al., 2011).

Based on our present study, the incorporation of HPN into SE primer showed the potential to improve the immediate bond strength and mechanical properties of resin-dentine interface. It is thought that the application of HPN in conjunction with dental adhesives is a promising approach to improve durability of bonds and lead to clinical success of adhesive restorations. Further studies are necessary to evaluate the long term performance of HPN and GSE on resin-dentine bond and other possible use of HPN in dentistry.

5. Conclusion

Within the limitations of our *in vitro* study, it can be concluded that HPN-incorporated self-etching primer improved the immediate bond strength and mechanical properties of resin-dentine interface; while GSE-incorporated primer significantly decreased the bond strength and mechanical properties. The CHX-incorporated group did not show any significant effect on these properties. It can be implied that HPN should have a potential to preserve the bonded interface, thereby improving the long-term bond durability.

Chapter 4

Effect of Hesperidin Incorporation into a Self-Etching Primer on Durability of Dentin Bond

Abstract

Introduction: Collagen degradation at the resin-dentin interface deteriorates dentin bond durability. The use of natural cross-linkers might offer a positive approach to stabilize the resin-dentin interface. **Objectives:** This study evaluated the effects of incorporation of natural cross-linkers into a self-etch adhesive primer on the immediate and long-term micro-tensile bond strengths (μTBS) to dentin. **Method:** Experimental primers were prepared by incorporating either 0.5%, 1%, 2%, 5% of hesperidin (HPN) or 0.5% of proanthocyanidins (PA) into Clearfil SE primer. Extracted human molar teeth were restored using the experimental primers or the pure SE primer (control). The mechanical properties of the bonded interfaces were measured using the nano-indentation tests. Beam-shaped bonded specimens were sub-divided for one-day and one-year μTBS test. Interfacial collagen morphology was observed using transmission electron microscopy. **Result:** The immediate μTBS significantly increased in 0.5%, 1% and 2% HPN-incorporated groups when compared with the control. The mechanical properties of bonded interface were improved with 1% and 2% HPN-incorporated primers. For the long-term μTBS, the 2% and 5% HPN-incorporated group were significantly higher than the control. The morphology of the collagen fibrils were preserved by 5% HPN-incorporation after one-year storage. The PA group, however, failed to improve the μTBS and the mechanical properties of the bonded interfaces. **Significance:** The incorporation of 2% HPN into the self-etching primer had a positive effect on the immediate μTBS and mechanical properties of the resin-dentin interfaces. The 5% HPN group preserved the morphology of the collagen in the hybrid layer after one-year storage in artificial saliva.

Key words: Micro-tensile bond strength, Hybrid layer, Hesperidin, Hardness, Elastic modulus, Collagen, Cross-linker.

1. Introduction

Adhesive restorations are widely distributed as the routine procedures in operative and restorative treatments. The use of self-etch adhesives became popular because it is less technique-sensitive, less aggressive to dentin, when compared with phosphoric-acid etching and shows less post-operative sensitivity (Van Meerbeek et al., 2011). However, deterioration of dentin bond occurs due to degradation in resin-dentin interface (Carrilho et al., 2005; Wang and Spencer, 2003; 2005; Yiu et al., 2004). Unlike etch-and-rinse technique, self-etch technique does not completely expose the collagen matrix (Van Meerbeek et al., 2003); however, the stability of collagen fibrils within the hybrid layer is crucial for the maintenance of bond effectiveness over time (Breschi et al., 2008; Liu et al., 2011). Past studies have focused on two major factors that can degrade resin-dentin interface. The first factor is the hydrophilic characteristic of the monomer that can be hydrolyzed in aqueous solution (Carvalho et al., 2004; De Munck et al., 2005; Nishitani et al., 2006a; Van Landuyt et al., 2008). The second factor is the uncured monomer that may remain at the bottom of the hybrid layer (Carrilho et al., 2005). These two phenomena consequently expose the collagen matrix that can be degraded by proteolytic enzyme (van Strijp et al., 2003). It has been reported that dentin collagenolytic and gelatinolytic activities can be suppressed by protease inhibitors (Pashley et al., 2004). Therefore, matrix metalloproteinase (MMPs) inhibitors such as chlorhexidine have been reported to be beneficial to preserve the hybrid layer and improve bond strength over time (Breschi et al., 2010). Recently, many researchers have reported that the use of collagen cross-linker to acid-etched dentin can prevent collagen degradation

within the hybrid layer and maintain good dentin bond strength (Liu et al., 2011). To simplify the use of cross-linker in clinical situations, cross-linkers should be incorporated directly into dental primer or adhesives (Green et al., 2010). This clinical procedure allows cross-linkers to remain in the hybrid layer for an extended period of time, thereby enhancing the degree of collagen cross-linking and bio-degradation resistance.

Hesperidin (HPN), hesperetin-7-O-rutinoside, is a flavonoid extracted from citrus fruits. The pharmacological properties and medicinal uses of HPN are associated with its wide range of benefits such as anti-inflammatory (Emim et al., 1994; Galati et al., 1994), analgesic (Martinez et al., 2011), anti-microbial (Kawaguchi et al., 2004), and anti-oxidant effects (Hirata et al., 2005). HPN is also capable of carcinogenesis inhibition (Miller et al., 2008), bone loss prevention (Horcajada et al., 2008) and inhibition of MMPs' proteolytic activities (Kamaraj et al., 2010). We first attempted to apply HPN in root caries model in a pH cycle study, where HPN showed the potential to prevent collagen degradation against proteolytic enzyme (Islam et al., 2012b). Considering its cross-linking effect on dentin bonding, we have incorporated HPN into a primer of self-etch adhesive system, which effectively increased the immediate resin-dentin bond strength (Islam et al., 2012a).

In the present study, we aimed to evaluate the effect of incorporation of different concentrations of HPN into the primer of a self-etch adhesive on micro-mechanical properties of resin-dentin interfaces, immediate and long-term resin-dentin bond strength. The null hypotheses tested were that the incorporation of HPN has no effects i) on the mechanical properties of resin-dentin interfaces and ii) on the immediate and long-term resin-dentin bond strength.

Group	Materials tested	pH
1	Pure Clearfil SE primer	2.0
2	Clearfil SE primer + 0.5% HPN	2.0
3	Clearfil SE primer + 1% HPN	2.0
4	Clearfil SE primer + 2% HPN	2.0
5	Clearfil SE primer + 5% HPN	2.0
6	Clearfil SE primer + 0.5% PA	2.0

Clearfil SE primer	Clearfil SE bond
•MDP	•MDP
•HEMA	•HEMA
•Dimethacrylate monomer	•Dimethacrylate monomer
•Water	•Micro filler
•Catalyst	•Catalyst

Table. 3 Study design and composition of SE primer and SE bond

2. Material and methods

2.1 Micro tensile bond strength testing

The teeth used in this study were collected after obtaining the patients' informed consent. The Human Research Ethics Committee of Tokyo Medical and Dental University, Japan reviewed and approved this study under the protocol number 725. Forty-eight freshly extracted non-carious human molar teeth were used for bond strength testing. A flat dentin surface was created perpendicular to the tooth's longitudinal axis using a slow-speed diamond saw (Isomet, Buehler Ltd., Lake Bluff, IL, USA) under water cooling to remove occlusal dentin. Smear layer was produced on each surface using #600 Silicon Carbide paper under water irrigation. Eight teeth per group were allocated for the following self-etching primers. HPN (hesperetin-7-O-rutinoside, Wako Pure Chemical Industries, Ltd,

Tokyo, Japan) or grape seed derived proanthocyanidins (PA) (proanthocyanidins, Kikkoman Biochemifa, Chiba, Japan) was added to Clearfil SE primer (Kuraray Noritake Dental Inc. Tokyo, Japan) to formulate the experimental primer groups (0.5%, 1%, 2%, 5% HPN and 0.5% PA (Table 3). The pH value of each experimental primer was measured with a digital pH meter (Twin pH B-211, HORIBA, Ltd. Kyoto, Japan). The original SE primer served as control. The dentin surfaces were conditioned with the primers according to the manufacturer's instructions, then Clearfil SE bond (Kuraray Noritake Dental Inc. Tokyo, Japan) was applied and light-cured for 10 sec (OPTILUX 501, Kerr corporation, CA, USA. light intensity 650mW/cm2). Composite resin (Clearfil AP-X, Kuraray Noritake Dental Inc. Tokyo, Japan) was placed on dentin surfaces incrementally up to 5 mm of thickness. Each increment was light-cured for 30 sec. After storage in de-ionized water at 37ºC for 24 h, the bonded teeth were sectioned longitudinally into serial slabs, and further sectioned to obtain (0.9mm x 0.9mm) composite-dentin beams. The beams were divided into two test groups. Half of the specimens from each group were used for immediate micro-tensile bond strength (μTBS) testing and the remaining half were stored in artificial saliva for one year at 37ºC. The artificial saliva contained (mM): $CaCl_2$ (0.7), $MgCl_2.6H_2O$ (0.2), KH_2PO_4 (4.0), KCl (30), NaN_3 (0.3), and HEPES buffer (20).

Bond strength testing was performed for specimens after one day and one year storage. The exact dimension of each beam was measured using a pair of digital calipers. Any specimen close to pulp horns was discarded due to inadequate size of dentin for tensile testing. The number of beams used for bond strength testing ranged from 12 to 16 per tooth. Each beam was stressed to failure under tension using a universal testing machine (EZ Test, Shimadzu Co. Kyoto, Japan) at a crosshead speed of 1 mm/min.

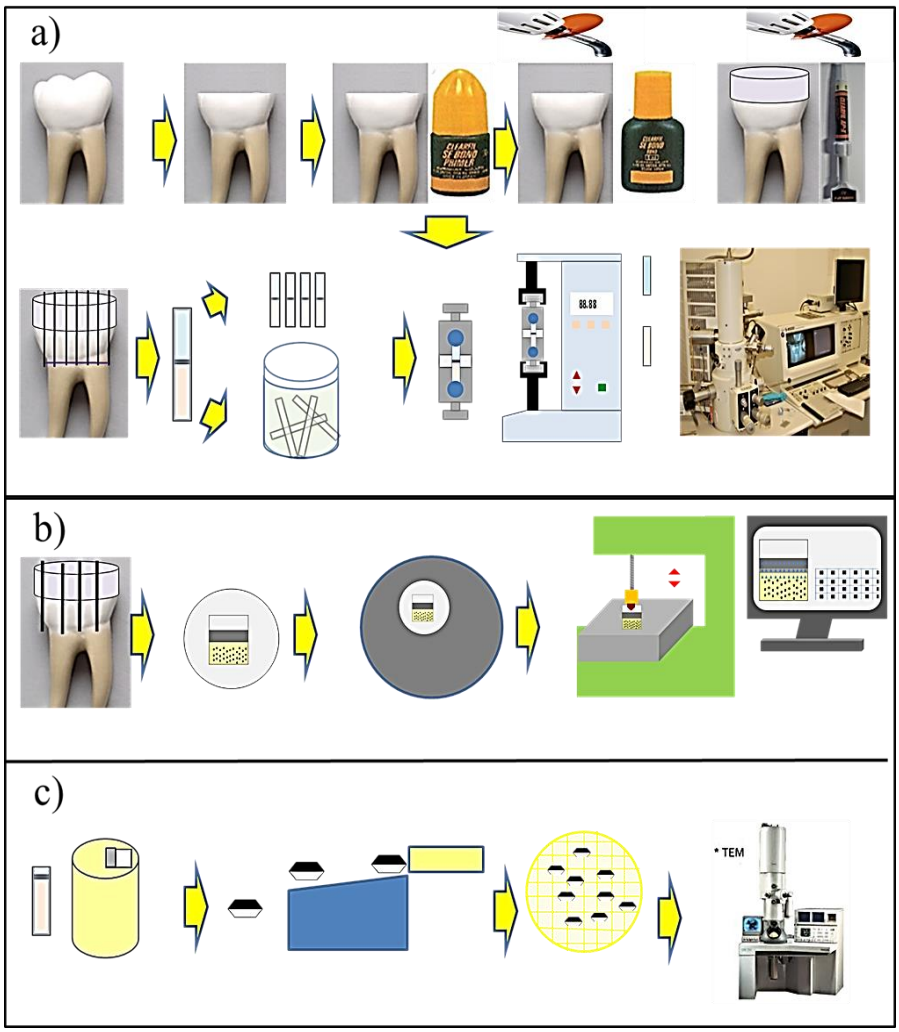

Fig. 11 Schema of a) Bond strength and failure mode, b) Mechanical properties c) Transmission electron microscopy

2.2 Failure mode

The fractured dentin surfaces were air-dried, sputter-coated with gold/palladium and examined using a scanning electron microscope (SEM, JSM-5310LV scanning microscope, JEOL Ltd. Tokyo, Japan) operating at 5 kV. The failure modes were categorized into four groups according to the type and

location, (A) mixed type of failure in resin composite and adhesive layer; (B) cohesive failure in adhesive layer; (C) mixed type of adhesive failure at the interface with retention of adhesive; (D) cohesive failure in dentin.

2.3 Examination of the etching effect of experimental primers on smear layer

To examine the effect of experimental SE primers on the smear layer, dentin discs of approximately 1 mm thickness were obtained from the mid-coronal dentin of another six extracted human third molars. The dentin surfaces were similarly treated with the pure primer or each of the experimental primers for 20 sec. Immediately after treatment, the discs were soaked in 100% acetone for 5 min to remove the applied primer, followed by dehydration in ascending concentrations of ethanol, then immersion in hexamethyldisilazane (Wako Pure Chemical Industries, Ltd, Tokyo, Japan) for 10 min and mounted on aluminum stubs and sputter-coated with gold/palladium and examined with the SEM operating at 5 kV.

2.4 Nano-indentation test

Dentin surfaces were prepared from 18 freshly extracted teeth. Three teeth in each group were bonded as previously described for tooth preparation. After storage in de-ionized water for 24 h at 37^{o}C, nano-indentation tests were conducted to measure Hardness (H) and Elastic Modulus (EM) of the adhesive layers and bonded interfaces. The test methodology was followed as described in our previous study (Islam et al., 2012a). Three slabs of 2 mm thickness were sectioned perpendicular to the bonding surface and selected from the center of each bonded specimen. The specimens were embedded in epoxy resin. After polishing with Silicon Carbide papers from #600 to #2000 and diamond pastes of decreasing particle sizes down to 0.25 μm, nano-indentation test was performed at

a constant temperature of 27.5°C with a Berkovich indenter attached to a computer-controlled nano-indentation device (ENT-1100, Elionix. Tokyo, Japan). The positions of indentation points were programmed at an interface, 10μm distance from the interface in the adhesive layer and in the dentin. Data of H were assigned to H_1 (hardness at the adhesive layer), H_2 (hardness at the interface) and H_3 (hardness at dentin). The relative hardness at the interface ($H_{2/3}$) was calculated as the ratio of H_2 to H_3 ($H_{2/3}=H_2/H_3$) to consider intrinsic difference in hardness of individual dentin substrate. The data of EM were determined in the same manner and assigned to EM_1, EM_2, EM_3 and $EM_{2/3}$. The data were obtained from the average value of 10 points of each location. Statistical analysis was performed for 9 specimens (3 slabs each from 3 bonded teeth) in each group.

2.5 Transmission electron microscopy (TEM)

The deboned specimens near the mean μTBS from each group were allocated for TEM examination. The dentin sides of beams were demineralized in an aqueous solution 15% (w/v) EDTA a pH of 7.0 for 7 days at room temperature. The specimens were fixed in a solution 2.5% glutaraldehyde for 2 h followed by 2% paraformaldehyde in 0.1 mol/L cacodylate buffer, (pH7.3) for 1 h finally in osmium tetroxide for 2 h. The specimens were dehydrated in increasing concentrations of ethanol (50%, 60%, 70%, 80%, 90% for 25 min and 100% for 20 min each). Then the specimens were embedded in epoxy resin at 60°C for 96 h. After resin embedding, ultra-thin transverse sections (ca.70 nm) were obtained with an ultra-microtome using a diamond knife and were collected onto 150 mesh copper grids under microscope (Sciences, Fort Washington, PA, USA). Specimens were double stained in 2% aqueous uranyl acetate for 20 min and Sato's lead citrate for 5 min. After drying, the sections were observed with TEM (H-7100; Hitachi, Tokyo, Japan) operating at 75 kV.

2.6 Statistical analysis

The data were analyzed using a statistical software package (Sigma Stat Version 16.0, SPSS, Chicago, IL, USA). The μTBS data were analyzed using two-way ANOVA, followed by one-way ANOVA and Tukey post hoc for multiple comparisons. The mechanical properties (H and EM) were analyzed using one-way ANOVA and Tukey post hoc for multiple comparisons. Level of statistical significance was set at 5%.

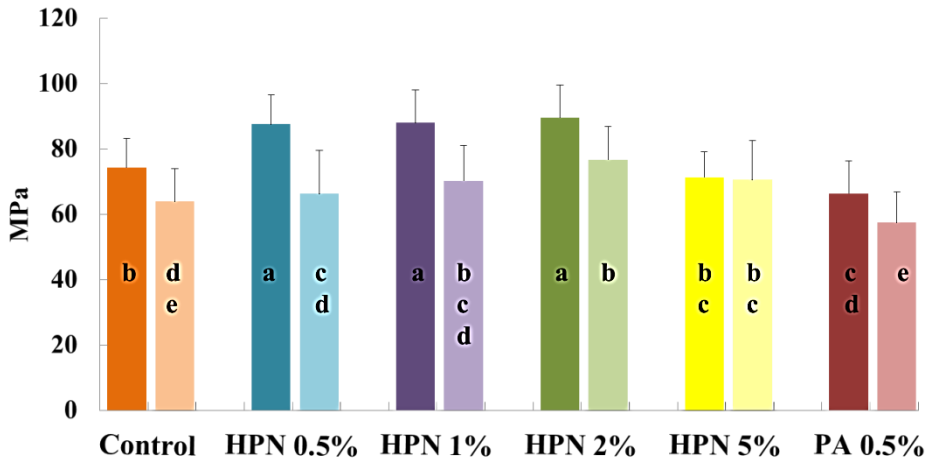

Fig. 12 Micro tensile bond strength values: Dark bar represents bond strength after 24 hrs water storage (n=52~59). Groups identified with same letters are not statistically significantly different ($p>0.05$).

3. Results

3.1 Micro tensile bond strength

The immediate and long-term bond strengths of all experimental groups are shown in Figure 12. Two-way ANOVA showed that the two factors “self-etching primers” and “time” had a significant effect on bond strength ($p<0.001$), and that

the interaction of the two factors was also significant ($p<0.001$). The 0.5% and 1% HPN-incorporated groups showed significantly higher immediate μTBS, when compared to the control group ($p<0.001$). For the long-term bond strength, there was no significant difference among the control groups, ($p>0.05$ put p value). The 2% HPN-incorporated group showed significantly higher immediate and long-term bond strengths when compared with the control ($p<0.001$). The 5% HPN-incorporated group did not show any significant difference in immediate μTBS when compared with the control ($p=0.928$). No significant difference was observed between the immediate and long-term bond strengths of the 5% HPN group ($p>0.05$). In contrast, all other groups showed significant reduction in μTBS after one-year storage in artificial saliva ($p<0.001$). The PA-incorporated group showed significantly lower immediate μTBS when compared to the control group ($p=0.018$); however, no significant difference in μTBS was found between the PA-incorporated and the control groups following aging ($p=0.077$).

3.2 Failure mode

The percentages of failures modes of specimens tested immediately and following aging in artificial saliva are shown in Figure 13. The failure patterns observed in the immediately tested specimens were mostly type A and type B. The 2% HPN-incorporated group showed a lower percentage of adhesive failures (type C); while a higher percentage of type C failure was observed in the PA-incorporated group. The number of type C failure increased remarkably in the one-year aged control specimens. By contrast, no remarkable change in failure pattern was observed between the immediately tested and one-year aged specimens of the 5% HPN-incorporated group.

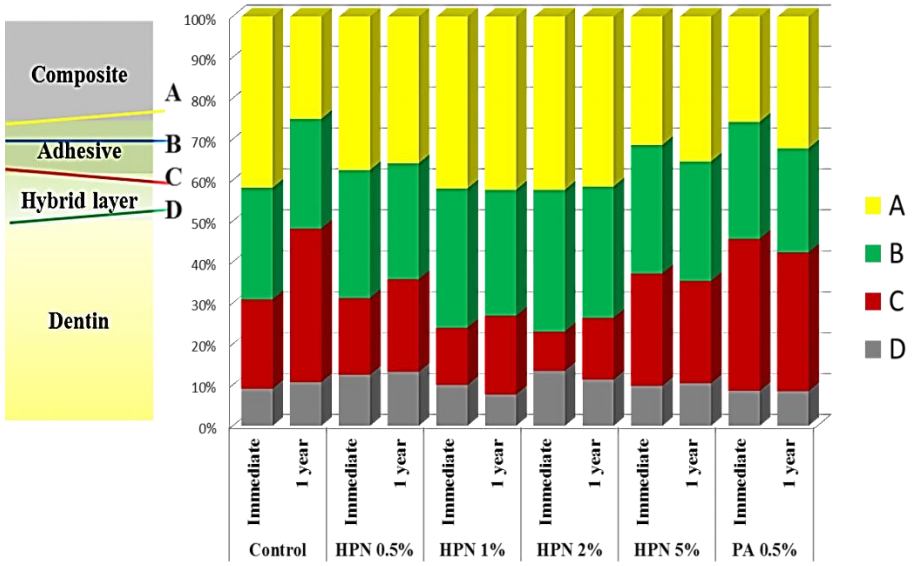

Fig. 13 Failure pattern; The left illustration shows the location of failure, and the right side chart shows the percentage of failure pattern according to the location. First bar show immediate failure patterns and second bar show those after one-year storage in artificial saliva.

3.3 Effect of experimental primers on smear layer

The pH of experimental primers and the observation of dentin surface under SEM did not show any remarkable differences among the groups. Open dentinal tubules were observed without any occluded smear particle in all tested groups, except the PA-incorporated group, where occlusions of dentinal tubules by smear-plugs were observed (Figure 15).

3.4 Nano-indentation

Table 4 summarizes the results of the mechanical properties of the bonded interfaces. One-way ANOVA revealed that the incorporation of HPN and PA showed a significant effect on the mechanical properties ($p<0.001$). Tukey post-hoc test revealed that the H ratio ($H_{2/3}$) of 2% HPN-incorporated group was significantly higher than control group ($p<0.01$) and 0.5% PA-incorporated group

was significantly lower than control group (p=0.037). Other experimental groups did not show any statistical significant difference with the control group (p>0.05). EM ($EM_{2/3}$) ratio of 1% and 2% HPN-incorporated group were significantly higher than the control group (p<0.01) and 0.5% The PA-incorporated group showed significantly lower value than the control group (p=0.004). Incorporation of HPN within the primer up to 2% did not show any statistical significant difference in H and EM of adhesive (H_1, EM_1) when compared with the control (p>0.05). By contrast, H and EM of adhesive (H_1, EM_1) were significantly reduced in 5% HPN-incorporated group and 0.5% PA-incorporated group (p<0.001).

Group	Hardness ratio ($H_2/H_{3)}$	Hardness of Adhesive (H_1)	Elastic modulus ratio ($EM_2/EM_{3)}$	Elastic modulus of Adhesive (EM_1)
1	60.5±1.7 ^{bc}	280.9±27.1 ^{a}	54.8±2.1 ^{cd}	6547±274 ^{a}
2	62.1±3.2 ^{bc}	277.4±7.7 ^{a}	58.2±2.5 ^{bc}	6483±322 ^{a}
3	63.2±3.6 ^{ab}	266.1±8.3 ^{a}	61.5±2.3 ^{b}	6421±91 ^{a}
4	66.1±2.2 ^{a}	262.6±8.8 ^{ab}	65.9±2.2 ^{a}	6348±189 ^{ab}
5	59.2±3.6 ^{cd}	247.6±12.7 ^{b}	54.3±2.3 ^{d}	6101±90 ^{b}
6	56.6±1.9 ^{d}	241.2±9.3 ^{c}	49.9±3.7 ^{e}	5801±172 ^{c}

Table. 4 Mechanical properties Hardness refers to the hardness ratio of interface with corresponding dentin, and H1 refers to the hardness of the adhesive. Elastic modulus refers to the elastic modulus ratio of interface with corresponding dentin, and E1 refers the to elastic modulus of the adhesive. Groups identified with same letters are not significantly different (p>0.05).

3.5 TEM observation

The representative images from the control, 2% HPN and 5% HPN-incorporated groups showed the morphological difference in collagen

matrix within each hybrid layer (Figure 16). TEM observation evaluated the morphological changes of collagen condition within the hybrid layer. Well-organized bands of collagen fibrils were observed within the hybrid layer of the specimens of 5% HPN-incorporated groups, whereas control group did not show such phenomenon. The control group showed denaturation of collagen fibers within the hybrid layer after one-year storage in artificial saliva. The 2% HPN-incorporated group showed well-organized collagen bands within the hybrid layer, but areas of degraded collagen bundles were also found in the same specimen. The 0.5% HPN-incorporated group and 1% HPN-incorporated group showed similar degradation patterns as the control (images not shown).

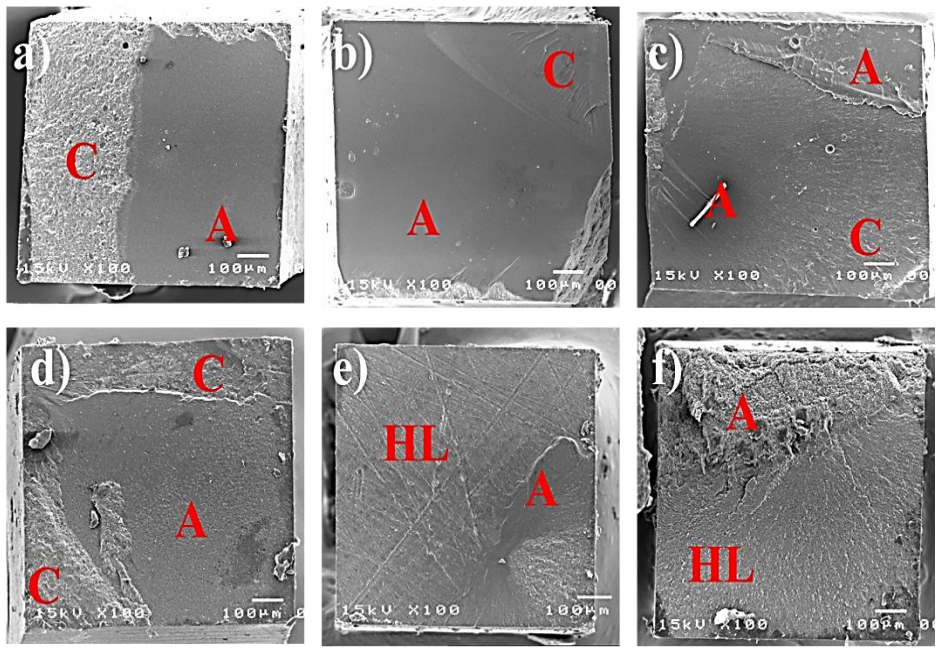

Fig. 14 Representative SEM images of fractured dentin surfaces a) control group; b) 0.5% HPN-incorporated group; c) 1% HPN-incorporated group; d) 2% HPN-incorporated group; e) 5% HPN-incorporated group; f) 0.5% PA-incorporated group.

4. Discussion

In the present study, the incorporation of 2% HPN into the SE primer showed significant improvements in the mechanical properties of resin-dentin interfaces, thus the first null hypothesis was rejected. The incorporation of 2% HPN significantly increased the immediate and long-term μTBS, when compared with the control, respectively. The 5% HPN showed the minimum reduction of μTBS after long-term storage, with no significant difference in the immediate μTBS between this group and the control. Thus the second null hypothesis was also rejected.

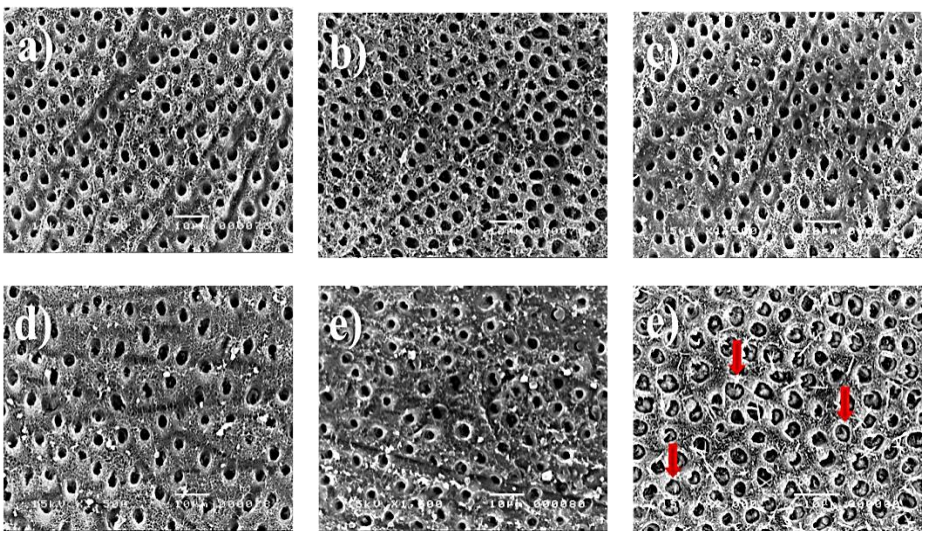

Fig. 15 SEM images of dentin surface after applying each experimental primer: a) Original SE primer; b) 0.5% HPN-incorporated primer; c) 1% HPN-incorporated primer; d) 2% HPN-incorporated primer; e) 5% HPN-incorporated primer; f) 0.5% PA-incorporated primer. The occluded smear plug shown with the ➡symbol

A wide range of biological activities of HPN has been reported, but the potential use of HPN in dentistry has not been well established. Our previous study revealed the efficacy of HPN to prevent proteolytic degradation of dentin

collagen (Hiraishi et al., 2011). HPN is a phenolic flavonoid, possessing a chroman ring similar to natural flavonoids. Therefore, the chemical action of HPN on collagen might be explained in the same manner as the cross-linking effect of other flavonoids. Several studies have reported the effect of natural cross-linkers on μTBS. Natural cross-liners such as tannic acid and PA, found in green tea and grape seeds respectively, were reported to improve resin-dentin bonding due to their collagen cross-linking properties (Bedran-Russo et al., 2009; Castellan et al., 2010; Green et al., 2010). In our study, we demonstrated a dose-dependent effect of HPN in SE primer on preservation of dentin collagen and on significant improvement of the immediate μTBS. This finding corresponds to the results of a previous study, in which collagen matrix was shown to contribute significantly to the tensile strength of dentin (Miguez et al., 2004). We also showed that one-year storage in artificial saliva significantly reduced the μTBS of all tested groups, except the 5% HPN-incorporated group, which did not show any significant reduction of μTBS. Stabilized collagen with reduced risk of proteolytic degradation by MMPs, may have preserved the long-term dentin bond strength (Hiraishi et al., 2011). The long-term μTBS were significantly reduced in all experimental groups when compared to their immediate values, when less than 5% HPN were incorporated in the self-etching primers. However, the μTBS of 2% HPN-incorporated group was significantly higher than the control group following one-year storage in artificial saliva.

Analysis of the micro-mechanical properties of resin-dentin interfaces by nano-indentation tests showed that incorporation of 2% HPN into self-etching primer significantly increased the EM of resin-dentin interface. The improved mechanical properties of resin-dentin interface might account for the high immediate μTBS obtained with 2% HPN-incorporated group. Also, SEM

observation of failure patterns revealed that the incorporation of 2% HPN contributed to a decrease in adhesive failure of the resin-dentin interfaces. This finding is consistent with the previous report that the percentage of adhesive failure is inversely proportional to the value of μTBS (Hashimoto et al., 2002). After one-year storage in artificial saliva, the adhesive failure percentage in the control group increased, which evidenced the degradation of resin-dentin interfaces.

The TEM observation of resin-dentin interfaces showed remarkable differences in the morphology of collagen among the control, 2% and 5% HPN-incorporated groups. The morphological degradation of interfacial collagen was shown in the control group; whereas the incorporation of 5% HPN in self-etching primer maintained the fibriler orientation of collagen, showing resistance to degradation. We speculate that HPN might have interacted with collagen fibrils and preserved their morphological structure.

The mechanism of interaction between natural cross-linkers and collagen fibrils has not completely understood. In drug-protein interaction theory, there are four types of non-covalent interactions between ligand and protein, i.e. hydrophobic effect, hydrogen bond, van der Waals force and electrostatic interaction (Zhou et al., 2012). Previous studies on HPN and protein such as human serum albumin-hesperidin and hemoglobin indicated that hydrophobic interaction played an important role between them (Ding et al., 2012a; Ding et al., 2012b). We perceive that hydrophobic interaction accounts for HPN-collagen interaction and cross-linking effect, which preserved collagen fibrils within hybrid layers as shown in TEM images of 5% HPN group. Further research is required to elucidate the behavior of HPN within resin-dentin interface and the molecular interaction with collagen fibrils.

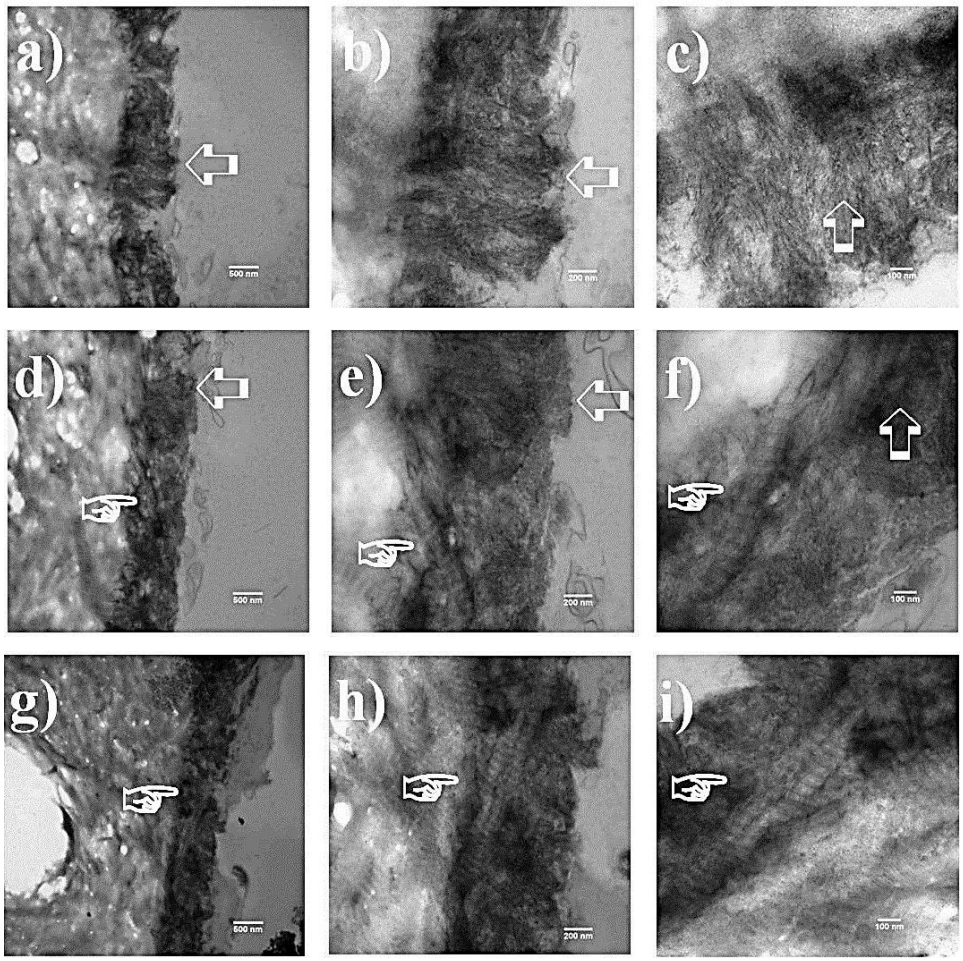

Fig. 16 Representative TEM images; a) Control group at 20,000x magnification; b) Control group at 50,000x magnification; c) Control group at 80,000x magnification; d) 2% HPN-incorporated group at 20,000x magnification; e) 2% HPN-incorporated group at 50,000x magnification; f) 2% HPN-incorporated group at 80,000x magnification; g) 5% HPN-incorporated group at 20,000x magnification; h) 5% HPN-incorporated group at 50,000x magnification; i) 5% HPN-incorporated group at 80,000x magnification. Area of denatured collagen within the hybrid layer showed by the ➪ symbol and well organized collagen bundle within the hybrid layer showed by the ☛ symbol.

Proanthocyanidins is a well-known cross-linker that has the potential to improve the immediate and long-term bond strength of pre-treated phosphoric acid-etched dentin (Al-Ammar et al., 2009); however, its incorporation into the SE primer failed to show such an effect in our present study. As we explained in our previous study, the large molecular size of PA might have hampered the etching effect of self-etch primer, thus inhibiting the ideal hybridization between the dentin and resin monomers (Islam et al., 2012b). A recent study showed a similar effect of PA-incorporated adhesive on dentin bond strength when PA was incorporated into adhesive resins (Epasinghe et al., 2012).

In the oral cavity, it has been considered that other factors, such as occlusal loading and thermal stress during function, would affect the durability of the bonded interface over time. It is very difficult to evaluate the actual bonding performance in clinical condition; however, the improvement in μTBS and mechanical properties of the bonded interface in our study may indicate the potential use of natural cross-linker, hesperidin in dentin bonding. Further *in vivo* studies are necessary to elucidate the optimal concentration of HPN to be incorporated into the self-etching primer for durable dentin bond.

5. Conclusion

Within the limitations of this study, we concluded that the incorporation of 2% HPN into the SE primer had superior performance than the pure SE primer on immediate μTBS, the mechanical properties of the bonded interface and long-term μTBS without compromising the properties of adhesive. However, 2% HPN incorporation revealed insufficient effect to prevent collagen degradation; whereas incorporation of 5% HPN into the SE primer can prevent interfacial collagen degradation and μTBS reduction after one-year storage in artificial saliva.

Chapter 5

General discussions and conclusions

Dentin is considered as the main bulk of the tooth. It is the bridge between the inorganic part (enamel) and organic part (pulp) of the tooth. Dentin is a complex mineralized tissue composed of approximately 70% mineral, 20% organic component and 10% fluid by weight. Type I collagen fiber accounts for 90% of the organic matrix (Ten Cate, 2008).

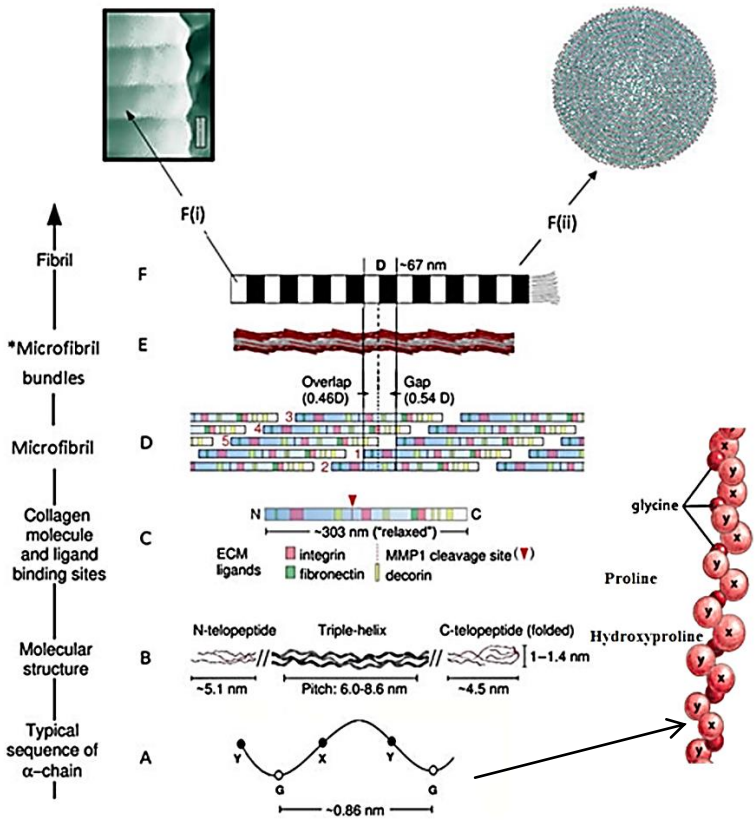

Fig. 17 Morphology of type-1 collagen; A) α chain B) collagen molecule C) binding sites of collagen molecule D) Micro fibril E) bundle of micro fibril F) collagen fibril Fi) AFM micrograph of a collagen fibril. F-ii) Lateral view of the molecular packing within a single fibril, where each circle represents the estimated position of each collagen molecule in cross-section. Collected from (Bertassoni et al., 2012)

Microscopically, we can observe the collagen fibril like a scaffold in dentin that is impregnated by the hydroxyl apatite crystals. The collagen fibril is a complex structure. The basic structure of collagen fibril starts with the α chain which consists of glycine, proline and 4-hydroxyprolinepraline. Each collagen molecule has three parts named as the N-terminal end, the C-terminal end and the triple helix that composed of 3α chains. The micro fibril structure is formed by assemblies of several collagen molecules. The association of several micro fibrils forms a bundle structure that is termed as collagen fibril (Bertassoni et al., 2012).

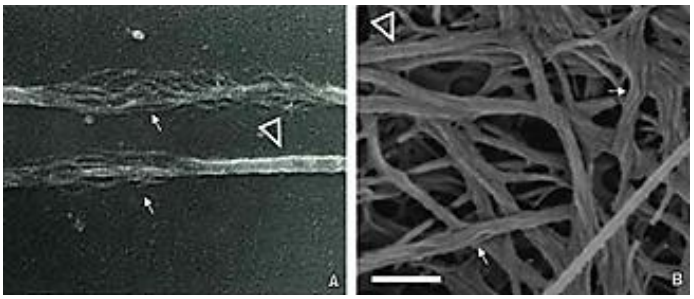

Fig. 18 Collagen fibril disaggregation unraveling thinner collagen internal sub structural units; (A) SEM image of corneal collagen fibrils treated with acetic acid. (B) Demineralized dentin collagen fibrils treated with trypsin yielding an untwisted rope like appearance. Collected from (Bertassoni et al., 2012)

The collagen fibrils play an important role both in caries formation and in adhesive restoration. It was recognized earlier that water plays an important role in maintaining the conformation of native collagen molecules (Fraser RDB, 1973). A variety of techniques and measurements such as nuclear magnetic resonance, dielectric measurements, water sorption and heat capacity indicated that water is either tightly bound to specific sites on collagen chains or fills the spaces between the collagen molecules (Cusack and Lees, 1984; Peto et al., 1990). This idea initially stemmed from the assertion that the specific register of the three

polypeptide chains in one collagen molecule relies on the presence of hydrogen bridges between Gly residues and the carboxyl oxygen (of an X (Pro) residue) from a neighboring chain (Bella et al., 1994). It has been reported that the lateral spacing between molecules is too long for direct hydrogen bonds between specific residues (Bella et al., 1994). It was confirmed thereafter that these inter-chain and inter-molecular bonds are formed by inherent water molecules which forms multi span hydrogen bonded bridges connecting neighboring collagen molecules (Bella et al., 1994). Based on these investigations, there is a general agreement that triple-helices are surrounded by a highly structured "cylinder of hydration" and the effective diameter of these "cylinders" dictates the lateral separation in the macromolecular assemblies that form the resulting fibriler units in type I collagen. Acid attacks either from bacteria or during etching for adhesive restoration create porosity and nano-voids at the fibril surface, where water molecules can be sequestered, leading to solvent uptake and elution (Pace and Datyner, 1980)

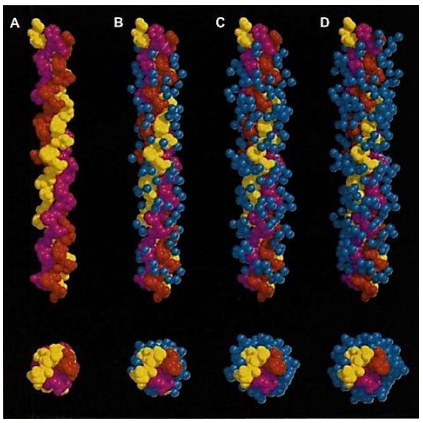

Fig. 19 Progressive hydration of the Gly-Ala peptide as seen in the crystal structure of the collagen molecule; each color represents one peptide chain of the triple helix, whereas the water molecules are shown in blue. (A) A view of the molecule without water incorporation (B) first, (C) second and (D) third shells of water molecules.

The host-derived proteinases make an important contribution to the degradation of exposed collagen, even when bacteria are not present (Carrilho et al., 2007a; Garcia-Godoy et al., 2007). A lot of studies described MMPs as a class of zinc and calcium-dependent endo-peptidases that are trapped within the mineralized dentin matrix during tooth development (Tjaderhane et al., 1998; van Strijp et al., 2003). The release and subsequent activation of these proteinases due to low pH are thought to be responsible for the degradation of collagen fibrils. An aspect that has received considerably less attention is the molecular packing arrangement of collagen and the micro-fibriler arrangement of the fibril surface. This controls the exposure of collagenolytic macromolecules (MMPs) binding sites. The currently accepted hypothesis (Perumal et al., 2008) is the fibrils might be damaged by the alteration of molecular arrangement such as the breakage of cross-linkages at the C-terminus. This may expose the catalytic binding site of the α2 chain that leads to the initial cleavage event. This initial cleavage, in turn, would facilitate the interaction of MMPs with other chains and trigger the collagenolytic process. For collagenolytic activity, structural changes within the collagen packing arrangement may be required for the binding of catalytic domain of the MMPs molecule to the targeted amino acid sequence of collagen molecule. This may be also valid for another type of collagenolytic enzymes in dentin that has been recently identified, namely cysteine cathepsins (Nascimento et al., 2011; Tersariol et al., 2010).

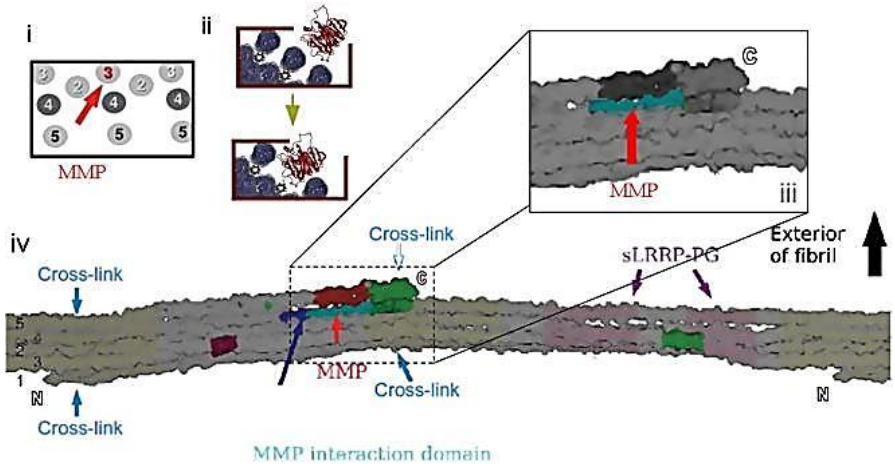

Fig. 20 MMP-1 as a model for MMP driven collagenolysis; (i) The MMP cleavage site is buried in a narrow cleft in the fibril surface. (ii) MMP access to collagen degradation is thought to require C-telopeptide removal for full enzyme access (top), as illustrated in the bottom image. Removal may not have to result in cleavage, however, it may be possible for the enzyme to squeeze into the cleft if the C-terminal region is moved due to extrinsic events affecting the packing arrangement of the fibril, such as in cases of thermal motion, bending of the fibril or putatively demineralization with strong acids, such as the phosphoric acid conditioner of adhesive systems. (iii) Longitudinal view of collagen molecular packing illustrating the MMP cleavage site (cyan) partially covered by the C-telopeptide region (green) (iv) The lower magnification view of (iii). Collected from (Bertassoni et al., 2012)

Cross-linking molecule can attach with the collagen molecule by hydrogen bond/ hydrophobic bond. Cross-linkage of the laterally packed molecules makes it more difficult for MMPs molecule to reach its α2 chain ‘‘target.’’ To alter the caries lesion first the damaged collagen should repair so that it can act as a scaffold for further mineral gain and revive the dentin structure. Most of the fundamental research on caries prevention focused on the re-mineralization of inorganic part. Some study also reported that application of MMP inhibitor can prevent collagen degradation. Some synthetic cross-linker like glutaraldehyde can

effectively fix the collagen to prevent degradation but their uses are limited for in vitro only. Plant derived natural extract like hesperidin and proanthocyanidins are biologically compatible with human body and works in the similar manner to prevent collagen degradation. Thus plant derived natural extracts (natural cross-linkers) enhance the re-mineralization process that can recover the early caries lesion and prevent further caries formation. Although fluoride application either systemic or topical is the most popular caries prevention therapy around the world, the re-mineralization mechanism and side effect of fluoride is not beyond the controversy. The novelty of our study is to introduce a new concept of caries prevention therapy by more bio-compatable natural extract.

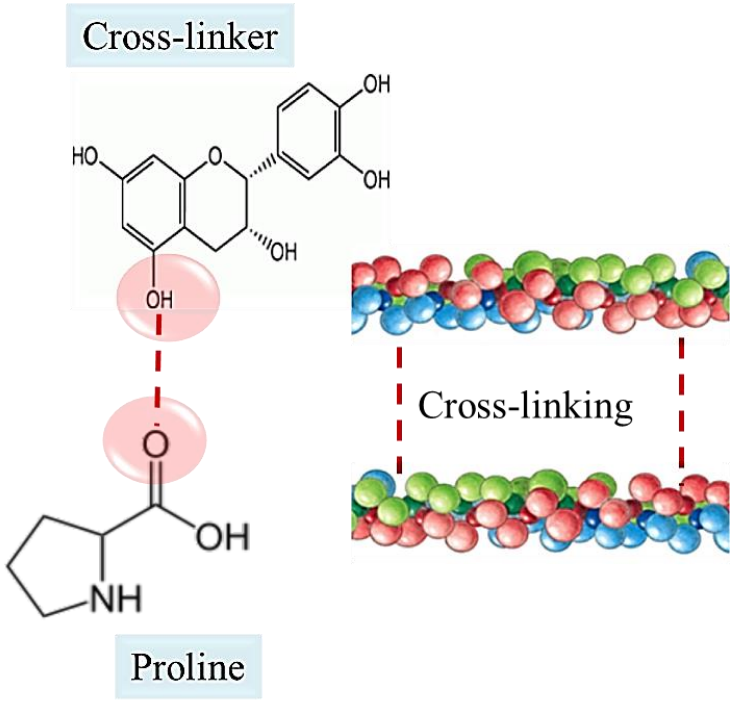

Fig. 21 schematic diagrams of cross-linker attachment with collagen molecule and cross link collagen molecule by hydrogen bond/ hydrophobic bond

For durable adhesive restoration preservation of interfacial collagen is an essential attempt. Failure of adhesive restoration could occur by the hydrolytic degradation of the resin-dentin interface. In our study we try to use the cross-linker in a dental adhesive system that can stay in the resin-dentin interface region. The hydrogen bond or hydrophobic bond between collagen and cross-linker gives additional strength to the collagen molecule to maintain the fibrillar orientation of collagen fibrils. Thus the cross-linker application can prevent the degradation of the resin-dentin interface. The novelty of our study is to establish a new strategy to reinforce the resin-dentin interface to achieve durable adhesive restoration.

According to the result of our consecutive study and within the limitation of in vitro study we can conclude that plant derived natural extract have the potential to reinforce caries prevention and improve the performance of adhesive restoration. Assuring the application of these natural extracts in regular dental care can improve the survival of natural teeth in short we can state that application of natural extract helps to achieve “super tooth”.

Chapter 6

References

References

Al-Ammar A, Drummond JL, Bedran-Russo AK (2009). The Use of Collagen Cross-Linking Agents to Enhance Dentin Bond Strength. *J Biomed Mater Res B* 91B(1)*:*419-424.

AR TC (1998). Oral Histology: development, structure and function: Idaho Falls: Lexington Book Inc.

Bedran-Russo AK, Yoo KJ, Ema KC, Pashley DH (2009). Mechanical properties of tannic-acid-treated dentin matrix. *J Dent Res* 88(9)*:*807-811.

Bedran-Russo AKB, Pereira PNR, Duarte WR, Drummond JL, Yamauchi M (2007). Application of crosslinkers to dentin collagen enhances the ultimate tensile strength. *J Biomed Mater Res B* 80B(1)*:*268-272.

Bella J, Eaton M, Brodsky B, Berman HM (1994). Crystal-Structure and Molecular-Structure of a Collagen-Like Peptide at 1.9-Angstrom Resolution. *Science* 266(5182)*:*75-81.

Benavente-Garcia O, Castillo J (2008). Update on uses and properties of citrus flavonoids: new findings in anticancer, cardiovascular, and anti-inflammatory activity. *J Agric Food Chem* 56(15)*:*6185-6205.

Bertassoni LE, Habelitz S, Kinney JH, Marshall SJ, Marshall GW (2009). Biomechanical Perspective on the Remineralization of Dentin. *Caries research* 43(1)*:*70-77.

Bertassoni LE, Orgel JPR, Antipova O, Swain MV (2012). The dentin organic matrix

- limitations of restorative dentistry hidden on the nanometer scale. *Acta Biomater* 8(7)*:*2419-2433.

Braga RR, Cesar PF, Gonzaga CC (2000). Tensile bond strength of filled and unfilled adhesives to dentin. *American journal of dentistry* 13(2)*:*73-76.

Breschi L, Mazzoni A, Ruggeri A, Cadenaro M, Di Lenarda R, Dorigo ED (2008). Dental adhesion review: Aging and stability of the bonded interface. *Dental Materials* 24(1)*:*90-101.

Breschi L, Martin P, Mazzoni A, Nato F, Carrilho M, Tjaderhane L *et al.* (2010). Use of a specific MMP-inhibitor (galardin) for preservation of hybrid layer. *Dental Materials* 26(6)*:*571-578.

Carrilho MR, Geraldeli S, Tay F, de Goes MF, Carvalho RM, Tjaderhane L *et al.* (2007a). In vivo preservation of the hybrid layer by chlorhexidine. *J Dent Res* 86(6)*:*529-533.

Carrilho MRD, Tay FR, Pashley DH, Tjaderhane L, Carvalho RM (2005). Mechanical stability of resin-dentin bond components. *Dental Materials* 21(3)*:*232-241.

Carrilho MRO, Carvalho RM, de Goes MF, di Hipolito V, Geraldeli S, Tay FR *et al.* (2007b). Chlorhexidine preserves dentin bond in vitro. *J Dent Res* 86(1)*:*90-94.

Carvalho RM, Pegoraro TA, Tay FR, Pegoraro LF, Silva NRFA, Pashley DH (2004). Adhesive permeability affects coupling of resin cements that utilise self-etching primers to dentine. *Journal of dentistry* 32(1)*:*55-65.

Castellan CS, Pereira PN, Grande RH, Bedran-Russo AK (2010). Mechanical characterization of proanthocyanidin-dentin matrix interaction. *Dental materials :*

official publication of the Academy of Dental Materials 26(10)*:*968-973.

Chaussain-Miller C, Fioretti F, Goldberg M, Menashi S (2006). The role of matrix metalloproteinases (MMPs) in human caries. *J Dent Res* 85(1)*:*22-32.

Choi EM, Kim YH (2008). Hesperetin attenuates the highly reducing sugar-triggered inhibition of osteoblast differentiation. *Cell biology and toxicology* 24(3)*:*225-231.

Clarkson BH, Rafter ME (2001). Emerging methods used in the prevention and repair of carious tissues. *Journal of dental education* 65(10)*:*1114-1120.

Cragg GM, Newman DJ, Snader KM (1997). Natural products in drug discovery and development. *J Nat Prod* 60(1)*:*52-60.

Cusack S, Lees S (1984). Variation of Longitudinal Acoustic Velocity at Gigahertz Frequencies with Water-Content in Rat-Tail Tendon Fibers. *Biopolymers* 23(2)*:*337-351.

de Castro FLA, de Andrade MF, Duarte SLL, Vaz JG, Ahid FJM (2003). Effect of 2% chlorhexidine on microtensile bond strength of composite to dentin. *J Adhes Dent* 5(2)*:*129-138.

De Munck J, Van Landuyt K, Peumans M, Poitevin A, Lambrechts P, Braem M *et al.* (2005). A critical review of the durability of adhesion to tooth tissue: Methods and results. *J Dent Res* 84(2)*:*118-132.

Dijkman GE, de Vries J, Arends J (1992). Effect of glutardialdehyde on secondary caries in situ. *Caries research* 26(4)*:*293-298.

Ding F, Diao JX, Sun Y, Sun Y (2012a). Bioevaluation of Human Serum

Albumin-Hesperidin Bioconjugate: Insight into Protein Vector Function and Conformation. *J Agr Food Chem* 60(29)*:*7218-7228.

Ding F, Sun Y, Diao JX, Li XN, Yang XL, Sun Y *et al.* (2012b). Features of the complex of food additive hesperidin to hemoglobin. *J Photoch Photobio B* 106(53-60.

dos Santos PH, Karol S, Bedran-Russo AKB (2011). Nanomechanical properties of biochemically modified dentin bonded interfaces. *Journal of oral rehabilitation* 38(7)*:*541-546.

Duarte S, Koo H, Bowen WH, Hayacibara MF, Cury JA, Ikegaki M *et al.* (2003). Effect of a novel type of propolis and its chemical fractions on glucosyltransferases and on growth and adherence of mutans streptococci. *Biol Pharm Bull* 26(4)*:*527-531.

Emim JA, Oliveira AB, Lapa AJ (1994). Pharmacological evaluation of the anti-inflammatory activity of a citrus bioflavonoid, hesperidin, and the isoflavonoids, duartin and claussequinone, in rats and mice. *The Journal of pharmacy and pharmacology* 46(2)*:*118-122.

Epasinghe DJ, Yiu CKY, Burrow MF, Tay FR, King NM (2012). Effect of proanthocyanidin incorporation into dental adhesive resin on resin-dentine bond strength. *Journal of dentistry* 40(3)*:*173-180.

Fontana M, Young DA, Wolff MS (2009). Evidence-based caries, risk assessment, and treatment. *Dental clinics of North America* 53(1)*:*149-161, x.

Fraser RDB MT (1973). Conformation in fibrous proteins. . *New York Academic Press*.

Frazier RA, Deaville ER, Green RJ, Stringano E, Willoughby I, Plant J *et al.* (2010).

Interactions of tea tannins and condensed tannins with proteins. *J Pharmaceut Biomed* 51(2)*:*490-495.

Fukuda Y, Nakashima S, Ujiie T (2009). The in vitro effect of a collagenolytic enzyme inhibitor on lesion development in root dentin. *American journal of dentistry* 22(2)*:*115-121.

Gaengler P, Hoyer I, Montag R, Gaebler P (2004). Micromorphological evaluation of posterior composite restorations - a 10-year report. *Journal of oral rehabilitation* 31(10)*:*991-1000.

Galati EM, Monforte MT, Kirjavainen S, Forestieri AM, Trovato A, Tripodo MM (1994). Biological effects of hesperidin, a citrus flavonoid. (Note I): antiinflammatory and analgesic activity. *Farmaco* 40(11)*:*709-712.

Garcia-Godoy F, Tay FR, Pashley DH, Feilzer A, Tjaderhane L, Pashley EL (2007). Degradation of resin-bonded human dentin after 3 years of storage. *American journal of dentistry* 20(2)*:*109-113.

Garcia MB, Carrilho MR, Nor JE, Anauate-Netto C, Anido-Anido A, Amore R *et al.* (2009). Chlorhexidine Inhibits the Proteolytic Activity of Root and Coronal Carious Dentin in vitro. *Caries research* 43(2)*:*92-96.

Gendron R, Grenier D, Sorsa T, Mayrand D (1999). Inhibition of the activities of matrix metalloproteinases 2, 8, and 9 by chlorhexidine. *Clin Diagn Lab Immun* 6(3)*:*437-439.

Green B, Yao XM, Ganguly A, Xu CQ, Dusevich V, Walker MP *et al.* (2010). Grape seed proanthocyanidins increase collagen biodegradation resistance in the dentin/adhesive interface when included in an adhesive. *Journal of dentistry*

38(11):908-915.

Griffin SO, Griffin PM, Swann JL, Zlobin N (2004). Estimating rates of new root caries in older adults. *J Dent Res* 83(8):634-638.

Guardia T, Rotelli AE, Juarez AO, Pelzer LE (2001). Anti-inflammatory properties of plant flavonoids. Effects of rutin, quercetin and hesperidin on adjuvant arthritis in rat. *Farmaco* 56(9):683-687.

Hara AT, Queiroz CS, Leme AFP, Serra MC, Cury JA (2003). In situ evaluation of caries progression and inhibition in bovine and human root dentin. *J Dent Res* 82(230-230.

Hara AT, Ando M, Cury JA, Serra MC, Gonzalez-Cabezas C, Zero DT (2005). Influence of the organic matrix on root dentine erosion by citric acid. *Caries research* 39(2):134-138.

Hashimoto M, Ohno H, Kaga M, Sano H, Tay FR, Oguchi H *et al.* (2002). Over-etching effects on micro-tensile bond strength and failure patterns for two dentin bonding systems. *Journal of dentistry* 30(2-3):99-105.

Hashimoto M, Ohno H, Sano H, Kaga M, Oguchi H (2003). In vitro degradation of resin-dentin bonds analyzed by microtensile bond test, scanning and transmission electron microscopy. *Biomaterials* 24(21):3795-3803.

Haslam E (1996). Natural polyphenols (vegetable tannins) as drugs: Possible modes of action. *J Nat Prod* 59(2):205-215.

Hebling J, Pashley DH, Tjaderhane L, Tay FR (2005). Chlorhexidine arrests subclinical degradation of dentin hybrid layers in vivo. *J Dent Res* 84(8):741-746.

Hiraishi N, Sono R, Islam MS, Otsuki M, Tagami J, Takatsuka T (2011). Effect of hesperidin in vitro on root dentine collagen and demineralization. *Journal of dentistry* 39(5)*:*391-396.

Hirata A, Murakami Y, Shoji M, Kadoma Y, Fujisawa S (2005). Kinetics of radical-scavenging activity of hesperetin and hesperidin and their inhibitory activity on COX-2 expression. *Anticancer Res* 25(5)*:*3367-3374.

Holm-Pedersen P, Schultz-Larsen K, Christiansen N, Avlund K (2008). Tooth loss and subsequent disability and mortality in old age. *Journal of the American Geriatrics Society* 56(3)*:*429-435.

Horcajada MN, Habauzit V, Trzeciakiewicz A, Morand C, Gil-Izquierdo A, Mardon J *et al.* (2008). Hesperidin inhibits ovariectomized-induced osteopenia and shows differential effects on bone mass and strength in young and adult intact rats. *J Appl Physiol* 104(3)*:*648-654.

Inaba D, Takagi O, Arends J (1997). A computer-assisted videodensitometric method to visualize mineral distributions in in vitro and in vivo formed root caries lesions. *European journal of oral sciences* 105(1)*:*74-80.

Islam S, Hiraishi N, Nassar M, Yiu C, Otsuki M, Tagami J (2012a). Effect of natural cross-linkers incorporation in a self-etching primer on dentine bond strength. *Journal of dentistry* 40(12)*:*1052-1059.

Islam SM, Hiraishi N, Nassar M, Sono R, Otsuki M, Takatsura T *et al.* (2012b). In vitro effect of hesperidin on root dentin collagen and de/re-mineralization. *Dental materials journal* 31(3)*:*362-367.

Kamaraj S, Anandakumar P, Jagan S, Ramakrishnan G, Devaki T (2010). Modulatory effect of hesperidin on benzo(a)pyrene induced experimental lung carcinogenesis with reference to COX-2, MMP-2 and MMP-9. *Eur J Pharmacol* 649(1-3)*:*320-327.

Kato MT, Leite AL, Hannas AR, Buzalaf MA (2010). Gels containing MMP inhibitors prevent dental erosion in situ. *J Dent Res* 89(5)*:*468-472.

Kawaguchi K, Kikuchi S, Hasunuma R, Maruyama H, Yoshikawa T, Kumazawa Y (2004). A citrus flavonoid hesperidin suppresses infection-induced endotoxin shock in mice. *Biol Pharm Bull* 27(5)*:*679-683.

Kawasaki K, Featherstone JD (1997). Effects of collagenase on root demineralization. *J Dent Res* 76(1)*:*588-595.

Kimura A, Arakawa H, Noda K, Yamazaki S, Hara ES, Mino T *et al.* (2012). Response shift in oral health-related quality of life measurement in patients with partial edentulism. *Journal of oral rehabilitation* 39(1)*:*44-54.

Liu Y, Li Y, Liu S, Li J, Yao S (2004). Monitoring the self-assembly of chitosan/glutaraldehyde/cysteamine/Au-colloid and the binding of human serum albumin with hesperidin. *Biomaterials* 25(26)*:*5725-5733.

Liu Y, Tjaderhane L, Breschi L, Mazzoni A, Li N, Mao J *et al.* (2011). Limitations in Bonding to Dentin and Experimental Strategies to Prevent Bond Degradation. *J Dent Res* 90(8)*:*953-968.

Lussi A, Linde A (1993). Mineral induction in vivo by dentine proteins. *Caries research* 27(4)*:*241-248.

Lynch E, Baysan A (2001). Reversal of primary root caries using a dentifrice with a

high fluoride content. *Caries research* 35 Suppl 1(60-64.

Macedo GV, Yamauchi M, Bedran-Russo AK (2009). Effects of Chemical Cross-linkers on Caries-affected Dentin Bonding. *J Dent Res* 88(12)*:*1096-1100.

Magalhaes AC, Wiegand A, Rios D, Hannas A, Attin T, Buzalaf MAR (2009). Chlorhexidine and green tea extract reduce dentin erosion and abrasion in situ. *Journal of dentistry* 37(12)*:*994-998.

Martinez AL, Gonzalez-Trujano ME, Chavez M, Pellicer F, Moreno J, Lopez-Munoz FJ (2011). Hesperidin produces antinociceptive response and synergistic interaction with ketorolac in an arthritic gout-type pain in rats. *Pharmacology, biochemistry, and behavior* 97(4)*:*683-689.

Mazzoni A, Pashley DH, Nishitani Y, Breschi L, Mannello F, Tjaderhane L *et al.* (2006). Reactivation of inactivated endogenous proteolytic activities in phosphoric acid-etched dentine by etch-and-rinse adhesives. *Biomaterials* 27(25)*:*4470-4476.

Mazzoni A, Mannello F, Tay FR, Tonti GA, Papa S, Mazzotti G *et al.* (2007). Zymographic analysis and characterization of MMP-2 and -9 forms in human sound dentin. *J Dent Res* 86(5)*:*436-440.

Miguez PA, Pereira PN, Atsawasuwan P, Yamauchi M (2004). Collagen cross-linking and ultimate tensile strength in dentin. *J Dent Res* 83(10)*:*807-810.

Miller EG, Peacock JJ, Bourland TC, Taylor SE, Wright JA, Patil BS *et al.* (2008). Inhibition of oral carcinogenesis by citrus flavonoids. *Nutr Cancer* 60(1)*:*69-74.

Nakabayashi N, Kojima K, Masuhara E (1982). The Promotion of Adhesion by the Infiltration of Monomers into Tooth Substrates. *J Biomed Mater Res* 16(3)*:*265-273.

Nascimento FD, Minciotti CL, Geraldeli S, Carrilho MR, Pashley DH, Tay FR *et al.* (2011). Cysteine Cathepsins in Human Carious Dentin. *J Dent Res* 90(4)*:*506-511.

Nishitani Y, Yoshiyama M, Donnelly AM, Agee KA, Sword J, Tay FR *et al.* (2006a). Effects of resin hydrophilicity on dentin bond strength. *J Dent Res* 85(11)*:*1016-1021.

Nishitani Y, Yoshiyama M, Wadgaonkar B, Breschi L, Mannello F, Mazzoni A *et al.* (2006b). Activation of gelatinolytic/collagenolytic activity in dentin by self-etching adhesives. *European journal of oral sciences* 114(2)*:*160-166.

Nostro A, Cannatelli MA, Crisafi G, Musolino AD, Procopio F, Alonzo V (2004). Modifications of hydrophobicity, in vitro adherence and cellular aggregation of Streptococcus mutans by Helichrysum italicum extract. *Lett Appl Microbiol* 38(5)*:*423-427.

Ooshima T, Osaka Y, Sasaki H, Osawa K, Yasuda H, Matsumura M *et al.* (2000). Caries inhibitory activity of cacao bean husk extract in in-vitro and animal experiments. *Archives of oral biology* 45(8)*:*639-645.

Opdam NJM, Loomans BAC, Roeters FJM, Bronkhorst EM (2004). Five-year clinical performance of posterior resin composite restorations placed by dental students. *Journal of dentistry* 32(5)*:*379-383.

Osorio R, Yamauti M, Osorio E, Ruiz-Requena ME, Pashley DH, Tay FR *et al.* (2011). Zinc reduces collagen degradation in demineralized human dentin explants. *Journal of dentistry* 39(2)*:*148-153.

Pace RJ, Datyner A (1980). Model of Sorption of Simple Molecules in Polymers. *J Polym Sci Pol Phys* 18(5)*:*1103-1124.

Pashley DH, Tay FR, Yiu C, Hashimoto M, Breschi L, Carvalho RM *et al.* (2004). Collagen degradation by host-derived enzymes during aging. *J Dent Res* 83(3)*:*216-221.

Perumal S, Antipova O, Orgel JPRO (2008). Collagen fibril architecture, domain organization, and triple-helical conformation govern its proteolysis. *P Natl Acad Sci USA* 105(8)*:*2824-2829.

Petersen PE (2005). Sociobehavioural risk factors in dental caries - international perspectives. *Community dentistry and oral epidemiology* 33(4)*:*274-279.

Petersson LG, Hakestam U, Baigi A, Lynch E (2007). Remineralization of primary root caries lesions using an amine fluoride rinse and dentifrice twice a day. *American journal of dentistry* 20(2)*:*93-96.

Peto S, Gillis P, Henri VP (1990). Structure and Dynamics of Water in Tendon from Nmr Relaxation Measurements. *Biophys J* 57(1)*:*71-84.

Reddy GK, Enwemeka CS (1996). A simplified method for the analysis of hydroxyproline in biological tissues. *Clinical biochemistry* 29(3)*:*225-229.

Ricci HA, Sanabe ME, Costa CAD, Hebling J (2010). Effect of chlorhexidine on bond strength of two-step etch-and-rinse adhesive systems to dentin of primary and permanent teeth. *American journal of dentistry* 23(3)*:*128-132.

Sasada M, Toichi M, Yamane H (2009). [Human health sciences]. *Nihon rinsho Japanese journal of clinical medicine* 67(1)*:*197-205.

Stanislawczuk R, Reis A, Loguercio AD (2011). A 2-year in vitro evaluation of a

chlorhexidine-containing acid on the durability of resin-dentin interfaces. *Journal of dentistry* 39(1)*:*40-47.

Sulkala M, Wahlgren J, Larmas M, Sorsa T, Teronen O, Salo T *et al.* (2001). The effects of MMP inhibitors on human salivary MMP activity and caries progression in rats. *J Dent Res* 80(6)*:*1545-1549.

Tay FR, Pashley DH, Loushine RJ, Weller RN, Monticelli F, Osorio R (2006). Self-etching adhesives increase collagenolytic activity in radicular dentin. *Journal of endodontics* 32(9)*:*862-868.

Ten Cate A (2008). Composition formation and structure of dentin.

ten Cate JM (2001). Remineralization of caries lesions extending into dentin. *J Dent Res* 80(5)*:*1407-1411.

Tersariol IL, Geraldeli S, Minciotti CL, Nascimento FD, Paakkonen V, Martins MT *et al.* (2010). Cysteine Cathepsins in Human Dentin-Pulp Complex. *Journal of endodontics* 36(3)*:*475-481.

Tjaderhane L, Larjava H, Sorsa T, Uitto VJ, Larmas M, Salo T (1998). The activation and function of host matrix metalloproteinases in dentin matrix breakdown in caries lesions. *J Dent Res* 77(8)*:*1622-1629.

Trzeciakiewicz A, Habauzit V, Mercier S, Barron D, Urpi-Sarda M, Manach C *et al.* (2010). Molecular Mechanism of Hesperetin-7-O-glucuronide, the Main Circulating Metabolite of Hesperidin, Involved in Osteoblast Differentiation. *J Agr Food Chem* 58(1)*:*668-675.

Van Landuyt KL, Snauwaert J, Peumans M, De Munck J, Lambrechts P, Van

Meerbeek B (2008). The role of HEMA in one-step self-etch adhesives. *Dental materials : official publication of the Academy of Dental Materials* 24(10)*:*1412-1419.

Van Meerbeek B, De Munck J, Yoshida Y, Inoue S, Vargas M, Vijay P *et al.* (2003). Buonocore Memorial Lecture - Adhesion to enamel and dentin: Current status and future challenges. *Oper Dent* 28(3)*:*215-235.

Van Meerbeek B, Yoshihara K, Yoshida Y, Mine A, De Munck J, Van Landuyt KL (2011). State of the art of self-etch adhesives. *Dental Materials* 27(1)*:*17-28.

van Strijp AJP, Jansen DC, DeGroot J, ten Cate JM, Everts V (2003). Host-derived proteinases and degradation of dentine collagen in situ. *Caries research* 37(1)*:*58-65.

Vanuspong W, Eisenburger M, Addy M (2002). Cervical tooth wear and sensitivity: erosion, softening and rehardening of dentine; effects of pH, time and ultrasonication. *Journal of clinical periodontology* 29(4)*:*351-357.

Walter R, Miguez PA, Arnold RR, Pereira PN, Duarte WR, Yamauchi M (2008). Effects of natural cross-linkers on the stability of dentin collagen and the inhibition of root caries in vitro. *Caries research* 42(4)*:*263-268.

Wang R, Zhou WB, Jiang XH (2008). Reaction kinetics of degradation and epimerization of epigallocatechin gallate (EGCG) in aqueous system over a wide temperature range. *J Agr Food Chem* 56(8)*:*2694-2701.

Wang Y, Spencer P (2003). Hybridization efficiency of the adhesive/dentin interface with wet bonding. *J Dent Res* 82(2)*:*141-145.

Wang Y, Spencer P (2005). Continuing etching of an all-in-one adhesive in wet dentin

tubules. *J Dent Res* 84(4)*:*350-354.

Wood N (2005). Bound sugars in hepatic glycoproteins from male rats during dietary citrus bioflavonoid and/or ascorbic acid supplementation. *Journal of medicinal food* 8(4)*:*512-517.

Xie Q, Bedran-Russo AK, Wu CD (2008). In vitro remineralization effects of grape seed extract on artificial root caries. *Journal of dentistry* 36(11)*:*900-906.

Yiu CKY, King NM, Pashley DH, Suh BI, Carvalho RM, Carrilho MRO *et al.* (2004). Effect of resin hydrophilicity and water storage on resin strength. *Biomaterials* 25(26)*:*5789-5796.

Zhang SC, Kern M (2009). The role of host-derived dentinal matrix metalloproteinases in reducing dentin bonding of resin adhesives. *International journal of oral science* 1(4)*:*163-176.

Zhou JF, Tan JG, Chen L, Li DL, Tan Y (2009). The incorporation of chlorhexidine in a two-step self-etching adhesive preserves dentin bond in vitro. *Journal of dentistry* 37(10)*:*807-812.

Zhou JF, Tan JG, Yang X, Xu XM, Li DL, Chen L (2011). MMP-Inhibitory Effect of Chlorhexidine Applied in a Self-etching Adhesive. *J Adhes Dent* 13(2)*:*111-115.

Zhou P, Huang J, Tian F (2012). Specific Noncovalent Interactions at Protein-Ligand Interface: Implications for Rational Drug Design. *Curr Med Chem* 19(2)*:*226-238.

Chapter 7

Biography

DR. Md. Sofiqul Islam

Cariology and Operative Dentistry

Tokyo Medical and Dental University

1-5-45, Yushima, Bunkyo-ku, Tokyo,

〒113-8549 Japan.

Tel: 0081 (0)3-5803-5483. Fax: 0081 (0)3-5803-0195

E-mail: soheltmd@gmail.com;

drsohel_bds2000@yahoo.com

Born 25th February 1982 at Bogra, Bangladesh

Education and work:

1997-1999: Higher Secondary School (HSC). Government Azizul Haque University College, Bogra, Bangladesh

2001-2006: Bachelor of Dental Surgery (BDS). Pioneer Dental College, University of Dhaka, Dhaka, Bangladesh

2006-2007: In-service training. Pioneer Dental College and Hospital, Dhaka, Bangladesh

2007: Post Graduate Training (PGT). Department of Orthodontics, Bangabandhu Sheikh Mujib Medical University Hospital (BSMMU) Dhaka, Bangladesh

2008-2009: Clinical training on Orthodontic under the fellowship of Japan Dental Association, Department of Orthodontics, Niigata University, Niigata, Japan

2010-2014: Doctorate of Philosophy (PhD) in Dental Science. Cariology and Operative Dentistry, Tokyo Medical and Dental University, Tokyo, Japan

Publications:

1. Effect of Hesperidin in vitro on Root Dentin Collagen and Demineralization. N. Hiraishi , R. Sono , M.S. Islam , M. Otsuki , J. Tagami , T. Takatsuka. J Dent. 2011 May; 39 (5): 391-6. Epub 2011 Mar 21
2. In vitro Effect of Hesperidin on Root Dentin Collagen and de/re-mineralization. Islam SM, Hiraishi N, Nassar M, Sono R, Otsuki M, Takatsura T, Yiu C, Tagami J. Dent Mater J. 2012; 31(3):362-7. Epub 2012 May 14
3. Effect of natural cross-linkers incorporation in a self-etching primer on dentine bond strength. Islam S, Hiraishi N, Nassar M, Yiu C, Otsuki M, Tagami J. J Dent. 2012 Dec; 40 (12): 1052-9. Epub 2012 Sep 11.
4. Effect of phytic acid used as etchant on bond strength, smear layer, and pulpal cells. Mohannad Nassar, Noriko Hiraishi, Md. Sofiqul Islam, Mamoru Aizawa, Yukihiko Tamura, Masayuki Otsuki, Shohei Kasugai, Keiichi Ohya and Junji Tagami . Article first published online: 20 JUN 2013 | DOI: 10.1111/eos. 12064
5. The effect of glutathione on 2-hydroxyethylmethacrylate cytotoxicity and on resin-dentine bond strength.Nassar M, Hiraishi N, Islam MS, Tamura Y, Otsuki M, Kasugai S, Ohya K, Tagami J, Tay FR.Int Endod J. 2013 Oct 9. doi: 10.1111/iej.12201.
6. In vitro evaluation of plant-derived agents to preserve dentin collagen. Hiraishi N, Sono R, Sofiqul I, Yiu C, Nakamura H, Otsuki M, Takatsuka T, Tagami J. Dent Mater. 2013 Oct;29(10):1048-54. doi: 0.1016/j.dental. 2013.07.015. Epub 2013 Aug 12.

Presentations:

1. **Islam Md. Sofiqul**, Hiraishi Noriko, Otsuki Masayoki, Tagami Junji. Effect of Hesperidin Incorporation into the Primer of a Self-etching Adhesive on Micro Tensile Bond Strength to Dentin.(Oral & poster presentations) The 4th International Congress on Adhesive Dentistry (April 15th -17th, 2011) Millennium Seoul Hilton hotel, Seoul, Korea
2. Hiraishi N, Sono R, **Islam MS**, Otsuki M, Tagami J, Takatsuka T. Effect of hesperidin in vitro on root dentine collagen and demineralization.(Oral presentation) The 89th General Session & Exhibition of the IADR (March 16th -19th, 2011) San Diego, Calif., USA
3. **Islam Sofiqul**, Hiraishi Noriko, Otsuki Hasayuki, Tagami Junji Effect of Grape Seeds on Stabilization of Collagen and Remineralization of the Root Dentin Lesion. (Oral presentation) The 134th meeting of Japanese society of conservative dentistry (June 9th -10th, 2011) Tokyo Disney resort Tokyo Bay Club Resort Hotel, Chiba, Japan.
4. **Islam Sofiqul**, Hiraishi Noriko, Otsuki Masayoki, Tagami Junji. Effect of Incorporation of Hesperidin in a Self-etching Primer on Dentin Bond Strength. (Oral presentation) The 135th meeting of Japanese society of conservative dentistry (October 20th -21st, 2011) Osaka international house foundation, Osaka, Japan.
5. **M.S. ISLAM**, N. HIRAISHI, M. NASSAR, M. OTSUKI, and J. TAGAMI. Effect of Cross-linker on Resin-dentin Bond Strength of Self-etch Adhesive. (Oral presentation) The 26th International Association of Dental Research South East Asia Division (November 3rd -4th, 2012), Hong Kong , China.
6. **Islam Sofiqul**, Hiraishi Noriko, Sono Ryohei, Otsuki Masayuki, Tagami Junji. In Vitro Evaluation of Plant-derived Agents to Preserve Dentin

Collagen. (Poster presentation) The 137th meeting of Japanese society of conservative dentistry (November 22nd -23rd , 2012) Hiroshima, Japan

7. NASSAR Mohannad, HIRAISHI Noriko, **Islam Md.Sofiqul**, OTSUKI Masayuki,TAGAMI Junji. Age-related changes in saliva and their effect on root caries. (Poster presentation) The 137th meeting of Japanese society of conservative dentistry (November 22nd -23rd, 2012) Hiroshima, Japan
8. Hiraishi Noriko, Kaneko Daisaku, Taira Shu, **Islam Sofiqul** , Otsuki Masayuki , Tagami Junji. Mussel-mimetic bio-adhesive polymers: the alternative to petroleum adhesives. The 137th meeting of Japanese society of conservative dentistry (November 22nd -23rd, 2012) Hiroshima, Japan
9. NASSAR Mohannad, HIRAISHI Noriko, **Islam Md.Sofiqul,** OTSUKI Masayuki, TAGAMI Junji, AIZAWA Mamoru. The Influence of Inositol Phosphate Used as Etchant on Resin-Dentin Bond Strength. (Oral presentation) The 31st Annual meeting of Japanese Society of Adhesive dentistry (December 8th-9th 2012) Tokyo Japan
10. Hiraishi Noriko, Kaneko Daisaku, Taira Shu, **Islam Sofiqul** , Otsuki Masayuki, Tagami Junji. Mussel-mimetic bio-adhesive resin: its application for dental adhesives. (Oral presentation), The 31st Annual Meeting of Japan Society of Adhesive Dentistry (December 8th -9th 2012) Tokyo, Japan,
11. **M.S. ISLAM**, N. HIRAISHI, Y. TAMURA, M. NASSAR, M. OTSUKI, S. KASUGAI, K. OHYA, S. TAIRA, D. KANEKO and J. TAGAMI. Biocompatibility of Mussel-mimetic Bio-adhesive Resin. (Oral presentation) IADR/AADR/CADR General Session. (March 20th – 23th, 2013). Washington Convention Center, Seattle, Washington. USA
12. M. NASSAR, N. HIRAISHI, Y. TAMURA, **M.S. ISLAM**, M. OTSUKI, S. KASUGAI, K. OHYA, J. TAGAMI1 and F.R. TAY. Glutathione

Detoxification of 2-hydroxyethylmethacrylate and Its Effect on Bond Strength. (Oral presentation) IADR/AADR/CADR General Session. (March 20th – 23th, 2013). Washington Convention Center, Seattle, Washington. USA

13. N. HIRAISHI, **M.S. ISLAM**, S. TAIRA, M. OTSUKI, J. TAGAMI, and D. KANEKO. Mussel-mimetic bio-adhesive polymers: the alternative to petroleum adhesives. (Poster presentation) IADR/AADR/CADR General Session. (March 20th – 23th, 2013). Washington Convention Center, Seattle, Washington. USA
14. **Islam S**, Kong K, Nassar M, Hiraishi N, Otsuki M, Tagami J. Etching effect of phytic acid on bond strength of MMA-based resin cement. (Oral presentation), The 32nd Annual Meeetin of Japan Society for Adhesive Dentistry. (30/11-1/12, 2013) Fukuoka, Japan
15. Kong K, **Islam S**, Nassar M, Hiraishi N, Otsuki M, Tagami J. Etching effect of phytic acid on dentin bond strength. (Oral presentation), The 32nd Annual Meeetin of Japan Society for Adhesive Dentistry. (30/11-1/12, 2013) Fukuoka, Japan

Awards:

1. Outstanding paper award; The 4th International Congress on Adhesive Dentistry (April 15-17, 2011) Millennium Seoul Hilton hotel, Seoul, Korea

2. IADR Geriatric Oral Research Awards; The 89th General Session & Exhibition of the IADR (March 16-19, 2011) San Diego, Calif., USA