JDAT

Journal of the Dental Association of Thailand

DFCT 2017

Supplement Issue

issn 0045 - 9917

วิทยาสารทันตแพทยศาสตร์
JOURNAL OF THE DENTAL ASSOCIATION OF THAILAND

Letter from President of Dental Association of Thailand

It is an honor for me to address in the opening chapter of this proceeding of the 15th National Scientific Conference of Dentistry (DFCT2017) .

Dental Faculty Consortium of Thailand (DFCT) has developed tremendous progresses in developing the effective curriculums and directives of dental education since the establishment in the Year 1983 (B.E. 2526).

On behalf of the President of Dental Association of Thailand I would like to express my sincere appreciation for all the efforts done by DFCT through the passing 34 years. Generation after generation of all Dental educators and administrators, DFCT has been placed at the forefront of their roles in creating thousands of newly qualified dental graduates with highly clinical proficiency for the Dental Society and for Thais.

The 15th National Scientific Conference of Dentistry is another big step of DFCT to promote the awareness and importance of regular participation in the Scientific conference. Thus will enable all the faculties to refresh and produce newly interesting topics in Dental Sciences and enhances the high capabilities of all faculty members, especially the young generation.

To conduct such an important event, the staffs and team work of the host are the crucial issue that will create success of the Conference. I would like to congratulate the Faculty of Dentistry, Naresuan University for the efforts done in creating this event. With highly energetic staffs and faculty members, this Conference will be the hall mark for all members of the Naresuan Universty.

Dr.Adirek S.Wongsa
President
Dental Association of Thailand

ทันตแพทยสมาคมแห่งประเทศไทย
ในพระบรมราชูปถัมภ์
THE DENTAL ASSOCIATION OF THAILAND

Advisory Board

Lt. Gen. Phisal	Thepsithar
Asst. Prof. Anonknart	Bhakdinaronk
Dr. Charmary	Reanamporn
Assoc. Prof. Porjai	Ruangsri
Assoc. Prof. Surasith	Kiatpongsan
Dr. Wantana	Puthipad
Dr. Werawat	Satayanurug
Prof. Dr. Waranun	Buajeeb
Dr. Prinya	Pathomkulmai

Board of Directors 2016 - 2018

President	Dr. Adirek	S.Wongsa
President Elect	Dr. Chavalit	Karnjanaopaswong
1st Vice-President	Asst. Prof. Dr. Sirivimol	Srisawasdi
2nd Vice-President	Assoc. Prof. Dr. Siriruk	Nakornchai
Secretary-General	Prof. Dr. Prasit	Pavasant
Treasurer	Assoc. Prof. Poranee	Berananda
Editor	Dr. Ekamon	Mahapoka
Executive Committee	Clinical Prof. Pusadee	Yotnuengnit
	Lt. Gen. Nawarut	Soonthornwit
	Assoc. Prof. Wacharaporn	Tasachan
	Dr. Somchai	Suthirathikul
	Asst. Prof. Ekachai	Chunhacheevachaloke
	Asst. Prof. Bundhit	Jirajariyavej
	Dr. Prae	Chittinand
	Dr. Kanit	Dhanesuan
	Assoc. Prof. Dr. Patita	Bhuridej
	Asst. Prof. Piriya	Cherdsatirakul
	Dr. Sutee	Suksudaj
	Assoc. Prof. Dr. Teerasak	Damrongrungruang

OFFICE 71 Ladprao 95 Wangtonglang Bangkok 10310, Thailand. Tel. 02-5394748, Fax 02-5141100

วิทยาสารทันตแพทยศาสตร์

JOURNAL OF THE DENTAL ASSOCIATION OF THAILAND

Journal published trimonthly. Foreign subscription rate US$ 200 including postage.
Publisher and artwork: Rungsilp Printing Co., Ltd
Please send manuscripts to Dr. Ekamon Mahapoka
Mailing address: 71 Ladprao 95 Wangtonglang Bangkok 10310, Thailand E-mail: jdat.editor@gmail.com

วิทยาสารทันตแพทยศาสตร์

JOURNAL OF THE DENTAL ASSOCIATION OF THAILAND

คณะอนุกรรมการฝ่ายวิชาการและคัดเลือกบทความ

วิทยาสารทันตแพทยศาสตร์
JOURNAL OF THE DENTAL ASSOCIATION OF THAILAND

คณะอนุกรรมการฝ่ายวิชาการและคัดเลือกบทความ

๑๒. ผู้แทนจาก คณะทันตแพทยศาสตร์ มหาวิทยาลัยพะเยา (ทันตแพทย์หญิง ศันสณี รัชชกูล)	กรรมการ
๑๓. ผู้แทนจาก สำนักวิชาทันตแพทยศาสตร์ มหาวิทยาลัยเทคโนโลยีสุรนารี (ศาสตราจารย์ เกียรติคุณ ทันตแพทย์หญิง ศิริพร ทิมปาวัฒน์)	กรรมการ
๑๔. หัวหน้าภาควิชาชีววิทยาช่องปาก (อาจารย์ ทันตแพทย์หญิง ดร.ฐานิต ประสิทธิ์ศักดิ์)	อนุกรรมการ
๑๕. หัวหน้าภาควิชาทันตกรรมบูรณะ (ผู้ช่วยศาสตราจารย์ ทันตแพทย์หญิง ดร.พีรยา ภูอภิชาติดำรง)	อนุกรรมการ
๑๖. หัวหน้าภาควิชาทันตกรรมป้องกัน (อาจารย์ ทันตแพทย์หญิง ดร.ประวีณา โสภาพรอมร)	อนุกรรมการ
๑๗. หัวหน้าภาควิชาศัลยศาสตร์ ช่องปาก (อาจารย์ ทันตแพทย์ พิชิต งามวรรณกุล)	อนุกรรมการ
๑๘. หัวหน้าภาควิชาทันตกรรมวินิจฉัย (รองศาสตราจารย์ ทันตแพทย์หญิง ดร.รัชดาภรณ์ เค้ามงคลกิจ)	อนุกรรมการ
๑๙. รองคณบดีฝ่ายวางแผนพัฒนาและประกันคุณภาพการศึกษา (อาจารย์ ทันตแพทย์ ดร.ชายแดน อินต๊ะปา)	อนุกรรมการและเลขานุการ
๒๐. อาจารย์ ทันตแพทย์ ดร. วรายุทธ โชติประกายเกียรติ	อนุกรรมการและผู้ช่วยเลขานุการ
๒๑. นางสาวศุภดา สวนนุ่ม	อนุกรรมการและผู้ช่วยเลขานุการ
๒๒. นางสาวชฎาภรณ์ บุญเนตร์	อนุกรรมการและผู้ช่วยเลขานุการ
๒๓. นางสาวเจนตา บัวเทศ	อนุกรรมการและผู้ช่วยเลขานุการ
๒๔. นางสาววราภรณ์ สุพระมิตร	อนุกรรมการและผู้ช่วยเลขานุการ

วิทยาสารทันตแพทยศาสตร์
JOURNAL OF THE DENTAL ASSOCIATION OF THAILAND

Contents

Volume 67 Supplement Issue 2017

วิทยาสารทันตแพทยศาสตร์

JOURNAL OF THE DENTAL ASSOCIATION OF THAILAND

Contents

Volume 67 Supplement Issue 2017

Original Article

Effects of Apacider® Mangostin Adhesive Pastes Combined with Fluoride Varnish on Remineralization Potential of Artificial Enamel Carious Lesions: *In vitro* Study

Pitchaya Puisuwan[1], Apa Juntavee[1], Niwut Juntavee[2], Waranuch Pitiphat[3], Jomjai Peerapattana[4] and Anoma Rattanacharoenthum[1]

[1]Department of Pediatric Dentistry, Faculty of Dentistry, Khon Kaen University, Khon Kaen

[2]Department of Prosthodontics, Faculty of Dentistry, Khon Kaen University, Khon Kaen

[3]Department of Community Dentistry and Chronic Inflammatory and Systemic Diseases Associated with Oral Health Research Group, Faculty of Dentistry, Khon Kaen University, Khon Kaen

[4]Department of Pharmaceutical Technology, Faculty of Pharmaceutical Sciences, Khon Kaen University, Khon Kaen

Abstract

Calcium phosphate-based technologies show promising efficacy as adjunctive treatment for fluoride therapy in the management of early carious lesions. Apacider® Mangostin Adhesive Pastes (AMAP) have been introduced as new alternative for the prevention and management of initial lesions. This study aimed to examine the effects of fluoride varnish combined with AMAP on remineralization of artificial enamel carious lesions in comparison to fluoride varnish alone, and AMAP alone. Artificial carious lesions were created on the buccal surfaces of 60 extracted premolars. Specimens were randomly divided into 5 groups: (1) fluoride varnish application at first day in combination with AMAP once a day continuously for 10 days, (2) fluoride varnish application at first day only, (3) AMAP application once a day continuously for 10 days, (4) blank AMAP application once a day continuously for 10 days, and (5) no treatment. All samples were treated under pH cycling for 10 days. Remineralization effect was evaluated by the difference in surface microhardness before and after agent application. There was a significant change in the mean surface microhardness before and after application in all groups ($p < 0.001$ each). A significant decrease in microhardness was observed in the fluoride varnish combined with AMAP group and fluoride varnish alone group, while a significant increase was found in AMAP alone group. The extent of the change was statistically different between AMAP group and fluoride varnish combined with AMAP group ($p < 0.001$). Comparison between fluoride varnish combined with AMAP group and fluoride varnish group showed no statistical difference ($p = 0.99$). In conclusion, AMAP has highest remineralization potential on artificial carious enamel lesions among all comparison groups. The application of fluoride varnish in combination with AMAP had no remineralization effect on artificial enamel carious lesions.

Keywords: Artificial caries, Fluoride varnish, Apacider® Mangostin Adhesive Pastes, Remineralization, Surface hardness

Received Date: APR 27, 2017 **Accepted Date:** JUL 17, 2017
doi: 10.14456/jdat.2017.16
Correspondence to:
Apa Juntavee. Department of Pediatric Dentistry, Faculty of Dentistry, Khon Kaen University, Amphur Muaeng, Khon Kaen 40002, Thailand
Tel: 043-202405 ext. 45157 Fax: 043-202862 E-mail: apa.edu@hotmail.com

Introduction

Non-cavitated carious lesions represents the initial carious lesions[1] caused by loss of calcium and phosphate ions from dissolution of hydroxyapatite crystal in the enamel structure.[2,3] Initial carious lesions can be arrested and reversed by remineralization.[1,4] New technologies for caries prevention and caries arrest have been developed in accordance with the minimally invasive intervention concept.[5,6] The philosophy includes early diagnosis of dental caries, assessment of individual caries risk, remineralization of early carious lesions, minimally surgical intervention of cavitated lesions with adhesive dental materials and repair rather than replacement of faulty restorations. The aim of remineralization strategy on initial carious lesions is to revert the lesions or to stop the progression,[7] to preserve remaining tooth structure,[5,6] as well as to improve strength, function and aesthetics.[2,7]

The effectiveness of fluoride on caries prevention was confirmed by many studies. It has also been proven to potentially arrest caries process.[8] If fluoride ions are presented in demineralized enamel surface, it can be absorbed to destroy apatite crystals and attracted calcium and phosphate ions to build new fluorapatites.[4] However, its ability to provide complete remineralization is limited by the availability of calcium and phosphate ions. Moreover, the high concentration of fluoride has the most effective remineralization effect on superficial surface of lesions. This is attributable to blocked crystal voids, which in turn reducing the penetration of ions to subsurface lesions resulting in incomplete remineralization.[7] Recently, a range of novel calcium phosphate-based technologies have been developed for clinical application[2] and show promising efficacy as adjunctive treatment for fluoride therapy in the management of early carious lesions.[9]

Apacider® AW (Sangi Co. Ltd., Tokyo, Japan) is an inorganic antimicrobial agent[10], based on apatite containing silver and zinc metals.[11] It enhances remineralizing activity from calcium-phosphate and antibacterial activity from silver ions.[12] An *in vitro* study of the effects of Apacider® varnish on surface microhardness and remineralization of enamel was performed by Juntavee *et al.* in 2009. The study showed significantly increased enamel microhardness and increased remineralization of white spot lesions at a level similar to fluoride varnish and CPP-ACP.[13] In 2015, Apacider® Mangostin Adhesive Pastes (AMAP) has been developed by Sodata and colleagues as a new alternative agent for the prevention and management of initial lesions with Apacider® AW as remineralizing agent and α-mangostin as antibacterial agent. An *in vitro* study showed that AMAP application can provide acid resistance and enhance consistent mineral gain during acid attack on artificial carious lesions.[12] Several previous studies have shown that remineralization of dental enamel could be enhanced by a sole application of AMAP[12] or fluoride varnish.[12-16] However, studies on synergistic remineralization effects of fluoride varnish application combined with AMAP on artificial enamel carious lesions have not been performed.

The objectives of this *in vitro* study were to

evaluate the effects of fluoride varnish combined with AMAP (FV-AMAP) in comparison to fluoride varnish only (FV), and AMAP only, on remineralization of artificial enamel carious lesions. Research hypothesis has been proposed on whether the application of fluoride varnish combined with AMAP has the remineralization effect on artificial carious enamel lesions

Materials and Methods

This *in vitro* study was conducted at the Faculty of Dentistry, Khon Kaen University. The study protocol was exempted from review by Khon Kaen University Ethics Committee in Human Research (HE592272).

1. Tooth specimen preparation

Sample size was calculated using Piface program[17] based on the study of Sodata,.[12] Sample size was calculated by. Using the formula for One-way ANOVA with a significance level of 5 %, power of 80 % and within-group standard deviation of 5.3 kgf/mm^2, a sample size of 10 specimens per group would be needed to detect a difference of 9.9 kgf/mm^2. Taking into account the possibly higher standard deviation in this study, 12 specimens were selected per group.

Sixty human lower premolars extracted for orthodontic treatment were collected. The tooth specimens were stored in 0.1 % thymol solution prior to the experiment. Calculus and soft tissue debris were removed using a 3/4 gracey curette. Specimens with carious lesions, crack, abrasion, enamel hypoplasia, fluorosis, tetracycline stain, or restorative material were excluded from this study. Tooth crown was separated from the root at cemento-enamel junction by using a tooth cutting machine (Mecatome T180, Brié-et-Angonnes, France) with a diamond-coated blade under water cooling. In order to prepare flat and smooth enamel surfaces, specimens were polished on the buccal surfaces with a polishing machine (Ecomet, Buehler, USA) using silicone carbide waterproof abrasive paper no. 1000, 1200, 1500, 2000 and 4000, respectively. Each specimen was coated with acid–resistant nail vanish (Revlon, New York, USA) except in a 4x4 mm^2 window area at the middle part of the buccal surface. The specimens were mounted in plastic blocks with buccal surface facing outward.

2. Artificial carious lesion formation

Sound tooth specimens were immersed in 5 mL of synthetic polymer gels, as described by Reynolds with minor modifications, for 12 hours at 37˚C to induce artificial carious lesions on buccal enamel.[18] The gel contained 20 g/L Carbopol™, 500 mg/L hydroxyapatite, 0.1 mol/L lactic acid, at pH 5.0.

3. Sample allocation and treatment protocol

Tooth specimens were randomly assigned into five groups (n = 12) as follows:

Group 1 Fluoride varnish (Duraphat®) application as recommended by the manufacturer on the first day in combination with AMAP for 3 minutes once daily continuously for 10 days (FV-AMAP)

Group 2 Fluoride varnish application as recommended by the manufacturer on the first day only (FV)

Group 3 AMAP application for 3 minutes once daily for 10 consecutive days (AMAP)

Group 4 Blank AMAP application for 3 minutes once daily for 10 consecutive days

Group 5 No treatment

The materials used and compositions are presented in Table 1.

4. Treatment of artificial carious lesions and pH cycling regimen

The solutions used in the pH-cycling model included: demineralizing solution, remineralizing solution and artificial saliva. Remineralizing and demineralizing solutions were prepared with analytical-grade chemicals and deionized water according to the method of Kumar *et al.*[19] for use in pH cycling. The demineralizing solution contains 2.2 mM $CaCl_2$, 2.2 mM KH_2PO_4, 0.05 M acetic acid and adjusted to pH 4.4 with 1 M KOH. The composition of remineralizing solution was 1.5 mM $CaCl_2$, 0.9 mM NaH_2PO_4, 0.15 M KCl to pH 7.0. Artificial saliva was prepared with 0.650 g/L KCl, 0.058 g/L $MgCl_2$, 0.165 g/L $CaCl_2$,

0.804 g/L, 0.365 g/L K_2HPO_4, 2.00 g/L C_6H_5COONa, 7.80 g/L $C_8H_{15}NaO_8$, and adjusted to pH 7.0. The pH-cycle solutions were freshly prepared daily. All tooth specimens were stored individually in 10 ml of solutions in scintillation vials. The specimens were subjected to pH-cycling system in a shaking water bath (Wisebath, Korea) for 10 cycles at 37°C. Each cycle was composed of demineralization for 3 hours twice a day with remineralization for 2 hours twice a day in between, and then the assigned agents were applied on the specimens by microbrushes. The specimens were left for 3 minutes after agent application, and then immersed in artificial saliva for 14 hours. The specimens were rinsed with deionized water after incubation with each solution. Demineralization and remineralization cycles are shown in Table 2.

Table 1 *List of materials used in the study*

Materials	Composition	Manufacturer
Fluoride varnish-Duraphat®	2.26 % sodium fluoride, ethanol, colophonium, mastix, shellac, wax, saccharine, flavor	Colgate-Palmolive, Hamburg, Germany
Apacider® Mangostin Adhesive Pastes	Apacider® AW, alpha-mangostin, fumed silica, Eudragit®, polyethylele glycol, 95 % ethyl alcohol, paraben	In patent of Khon Kaen University, Thailand
Blank Apacider® Mangostin Adhesive Pastes	Fumed silica, Eudragit®, polyethylele glycol, 95 % ethyl alcohol, paraben	In patent of Khon Kaen University, Thailand

Table 2 *The pH-cycling regimen in the experiment*

Time	Duration	Experimental solution
11.00 am - 2.00 pm	3 hours	Demineralizing solution
2.00 pm - 4.00 pm	2 hours	Remineralizing solution
4.00 pm - 7.00 pm	3 hours	Demineralizing solution
7.00 pm - 9.00 pm	2 hours	Remineralizing solution
9.00 pm – 11.00 am	3 minute	Testing agents
	14 hours	Submerged in artificial saliva

5. Assessment of surface microhardness (SMH)

The measurements of SMH were performed at baseline (before lesion formation), before agent application (after lesion formation) and after agent application using a microhardness tester (Future-Tech FM Corporation, Japan) with a Vickers diamond indenter giving force of 100 g for 5 seconds. The represented figure of indentation mark was shown on FT-ARS software version 1.15.13 (Future-Tech Corporation, Japan). SMH values were automatically calculated in this software. The average SMH was calculated and recorded as mean SMH before agent application (SMH1) and mean SMH after agent application (SMH2). Mean change in SMH (ΔSMH) was calculated by the formula: ΔSMH = SMH1-SMH2. Surface microhardness testing and indentation mark on enamel surface were shown in Fig. 1.

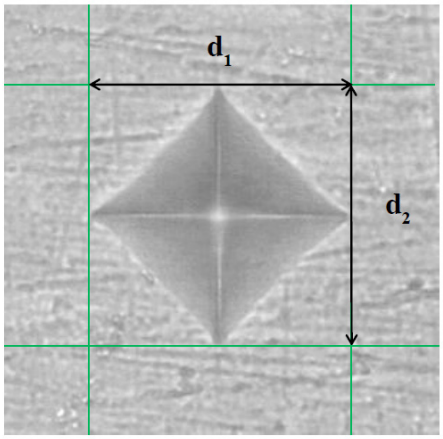

Figure 1 *Indentation mark on enamel surface (d1 and d2 are the diagonal length of indentation in mm.)*

Results

Means ± standard deviations (SD) of SMH before and after agent application are shown in Table 3. Before lesion formation, mean baseline SMH was 398.9 ± 9.9 kgf/mm^2 with no statistically significant differences among the five groups (p = 0.58). After lesion formation and before the agent application, the mean SMH was 178.6 ± 9.9 kgf/mm^2 with no statistically significant differences among the five groups (p = 0.35). There was a significant difference in SMH before and after application in each group (p < 0.001). FV-AMAP and FV groups had a decrease in SMH after agent application. In contrast, AMAP application resulted in an increase in SMH. The extent of the change was significantly different between AMAP and FV-AMAP groups (p < 0.001). However, there was no significant difference between FV-AMAP and FV groups (p = 0.99). The mean change in SMH in each group is shown in Table 4.

Table 3 *Mean and standard deviation (mean, SD) of surface microhardness before (SMH1) and after (SMH2) agent application*

Groups	Number of specimen	SMH1 (kgf/mm^2)	SMH2 (kgf/mm^2)	p-value[a]
FV-AMAP	12	174.5 ± 11.0	155.7 ± 12.4	< 0.001[b]
FV	12	181.7 ± 7.7	161.6 ± 14.3	< 0.001[b]
AMAP	12	180.1 ± 8.2	197.6 ± 11.3	< 0.001[b]
Blank AMAP	12	180.4 ± 10.4	89.3 ± 12.0	< 0.001[b]
No treatment	12	176.4 ± 11.2	84.1 ± 13.5	< 0.001[b]

[a] *Paired sample t-test, bSMH1 and SMH2 are significant difference (p < 0.001).*

FV-AMAP = Fluoride varnish combined with Apacider® Mangostin Adhesive Pastes; FV = Fluoride varnish; AMAP = Apacider® Mangostin Adhesive Pastes

Table 4 *Mean change in surface microhardness (ΔSMH) in each group*

Group	ΔSMH	95% Confidence Interval	
	(kgf/mm^2)	Lower bound	Upper bound
FV-AMAP	-18.8 ± 6.3[a]	-22.8	-14.8
FV	-20.1 ± 8.5[a]	-25.5	-14.7
AMAP	17.6 ± 6.4[b]	13.5	21.6
Blank AMAP	-91.1 ± 7.0[c]	-95.6	-86.7
No treatment	-92.3 ± 10.2[c]	-98.8	-85.8

[a, b, c] *Different superscript letters indicate statistically significant differences (One-way ANOVA, Tukey's test, P < 0.05)*

Discussion

To our knowledge, this study was the first *in vitro* study to evaluate the remineralization effect of fluoride varnish combined with AMAP using surface microhardness measurement. The microhardness test examines structural changes and the degree of mineralization of a substrate, especially after different treatments on dental enamel surfaces in unbalanced situations.[20,21] The Vickers microhardness test was chosen for our study due to its efficiency on the small round area and because it requires less enamel surface preparation.[21] The SMH baseline (398.9 kgf/mm^2) of this study corresponds to the average microhardness value of sound human enamel[22] of 370-420 kgf/mm^2

Artificial enamel carious lesion can be prepared by various techniques for the remineralization assessment of therapeutic agents. In other studies, the lesions were prepared by lactate or acetate gel at pH 4.4-5.0, to simulate organic acid produced by cariogenic bacteria. However, to mimic *in vivo* carious lesion, there should be subsurface lesion with less demineralized surface layer.[23] Therefore, we used synthetic polymer gels, composed of polyacrylic acid (Carbopol™), lactic acid and hydroxyapatite, for artificial caries formation.[18] The polyacrylic acid was supplemented as the main factor to preserve the surface layer and produce *in vitro* subsurface caries formation.[24] In the pH cycling model to simulate pH condition in an oral environment with dynamic mineral loss and gain, remineralizing agents were applied in the model for 10 days.[23] The application of fluoride varnish was designed to mimic clinical situation under the manufacturer's recommendation; however, in FV-AMAP group the fluoride varnish was applied on the first day followed by AMAP application until the end of ten days of the experimental cycle.

The results of this study showed partial remineralization on artificial enamel carious lesion which was possibly due to calcium and phosphate ions deficiency.[1] The deficiency of ions was attributed to the dissolution of hydroxyapatite crystal during artificial carious lesions formation, therefore, fluorapatites and calcium fluoride (CaF_2) synthesis was interrupted.[25] Partial remineralization was also found in FV-AMAP application. This may result from the hypermineralization of the surface layer physically blocks subsequent ingress of calcium and phosphate ions into subsurface layer of the lesion.[26] In contrast, AMAP application alone was able to provide calcium and phosp hate ions[12] that simultaneously diffuse into subsurface lesion for molecular structure restoration[27]. Similarly, Sodata's study in 2015 also observed homogeneous remineralization of artificial carious lesions after AMAP application.[12] Porosity and depth of artificial carious lesion play a critical role in mineral diffusion. Larger

porosities can induce higher mineral deposition, however, deeper lesions with longer distance can create difficulty for mineral ion absorption.[23] The results of AMAP group can be explained that small molecular structure of calcium and phosphate ions could be absorbed into deeper enamel porosity and deposited significantly larger amount of mineral than fluoride varnish counterpart as reflected by the higher values of surface SMH.[2,28]

The result from a previous study showed that fluoride varnish enhanced remineralization process of artificial carious lesions after agent application under the same pH cycling regimen as used by this study.[12] However, with different chemical formula for artificial carious lesion formation used in this study, it was shown that surface SMH values after fluoride varnish application were not increased. In our study, the reduction of SMH values after lesion formation was higher than in the previous study, indicating that the lesions have more progressive dissolution of apatite crystals or higher calcium phosphate ion depletion.

Our study found that fluoride varnish can only provide net mineral gain at a very early carious enamel lesion and reveal less acid tolerance comparing to AMAP. The application of AMAP alone showed superior effects on surface SMH after pH cycling in deeper carious enamel lesion than fluoride varnish and fluoride varnish combined with AMAP.

Conclusion

The results suggest that ten days' application of once daily-AMAP has the most effective remineralization potential on artificial carious enamel lesions when compared with other groups. The application of fluoride varnish application at first day in combination with AMAP once daily-continuously for 10 days has no significant remineralization effect on artificial carious enamel lesions.

Acknowledgement

We would like to thank Dr. Patteera Sodata for her help in preparing the testing agents and useful suggestion of laboratory technique, and Dr. Oranart Matangkasobut for suggestions to improve the manuscript. The financial support of the research was from Faculty of Dentistry, Khon Kean University.

References

1. Young DA, Novy BB, Zeller GG, Hale R, Hart TC, Truelove EL, *et al.* The American Dental Association Caries Classification System for clinical practice: a report of the American Dental Association Council on Scientific Affairs. ***J Am Dent Assoc*** 2015;146:79-86.

2. Cochrane N, Cai F, Huq N, Burrow M, Reynolds E. New approaches to enhanced remineralization of tooth enamel. ***J Dent Res*** 2010;89:1187-97.

3. Cummins D. Dental caries: a disease which remains a public health concern in the 21st century. The exploration of a breakthrough technology for caries prevention. ***J Clin Dent*** 2013;21:25-37.

4. Featherstone J. Dental caries: a dynamic disease process. ***Aust Dent J*** 2008;53:286-91.

5. Murdoch-Kinch CA, Mclean ME. Minimally invasive dentistry. ***J Am Dent Assoc*** 2003;134:87-95.

6. Azizi Z. Management of white spot lesions using resin infiltration technique: a review. ***J Dent Oral Med*** 2015;3:1-6.

7. Amaechi BT. Remineralization therapies for initial caries lesions. ***Curr Oral Health Rep*** 2015;2:95-101.

8. Kühnisch J, Ekstrand K, Pretty I, Twetman S, Van Loveren C, Gizani S, *et al.* Best clinical practice guidance for management of early caries lesions in children and young adults: an EAPD policy document. ***Eur J Paediatr Dent*** 2016;17:3-12.

9. Reynolds E. Calcium phosphate-based remineralization systems: scientific evidence? ***Aust Dent J*** 2008;53:268-73.

10. Sangi Co. Ltd. Technical data calcium phosphate

antimicrobial agents APACIDER AW- N. Tokyo; 2015.
11. Syafiuddin T, Hisamitsu H, Toko T, Igarashi T, Goto N, Fujishima A. *In vitro* inhibition of caries around a resin composite restoration containing antibacterial filler. ***Biomaterials*** 1997;18:1051-7.
12. Sodata P. The development of Apacider®-AW and alpha-mangostin adhesive Pastes for dental carien prevention and therapeutic approach [dissertation]. Khon Kaen; Khon Kaen University, Faculty of Pharmaceutical Sciences; 2015.
13. Juntavee A, Juntavee N, Luengpailin S, Chatrchaiwiwatana S, Suksupeaw S. Effects of Apacider Varnish on Microhardness of Human Dental Enamel. ***KKU Res J*** 2011;16:517-27.
14. Dehailan LA, Martinez-Mier EA, Lippert F. The effect of fluoride varnishes on caries lesions: an *in vitro* investigation. ***Clin Oral Investig*** 2016;20:1655-62.
15. Lata S, Varghese N, Varughese JM. Remineralization potential of fluoride and amorphous calcium phosphate-casein phospho peptide on enamel lesions: An in vitro comparative evaluation. ***J Conserv Dent*** 2010;13:42-6.
16. Mohd Said SN, Ekambaram M, Yiu CK. Effect of different fluoride varnishes on remineralization of artificial enamel carious lesions. ***Int J Paediatr Dent*** 2016;27:163-73.
17. Lenth RV. Java Applets for Power and Sample Size [Computer software] 2006 [cited 2016 Jul 1]. Available from: http://www.stat.uiowa.edu/~rlenth/Power.
18. Reynolds E. Remineralization of enamel subsurface lesions by casein phosphopeptide-stabilized calcium phosphate solutions. ***J Dent Res*** 1997;76:1587-95.
19. Kumar V, Itthagarun A, King N. The effect of casein phosphopeptide-amorphous calcium phosphate on remineralization of artificial caries-like lesions: an in vitro study. ***Aust Dent J*** 2008;53:34-40.
20. Tuloglu N, Bayrak S, Tunc ES, Ozer F. Effect of fluoride varnish with added casein phosphopeptide-amorphous calcium phosphate on the acid resistance of the primary enamel. ***BMC Oral Health*** 2016;16: 103-9.
21. Wang L, D'Alpino PHP, Lopes LG, Pereira JC. Mechanical properties of dental restorative materials: relative contribution of laboratory tests. ***J Appl Oral Sci*** 2003;11:162-7.
22. Fejerskov O, Kidd E, editors. Dental caries: the disease and its clinical management. 7th ed. Copenhagen: Blackwell Munksgaard; 2015. p. 211.
23. Buzalaf MAR, Hannas AR, Magalhães AC, Rios D, Honório HM, Delbem ACB. pH-cycling models for *in vitro* evaluation of the efficacy of fluoridated dentifrices for caries control: strengths and limitations. ***J Appl Oral Sci*** 2010;18:316-34.
24. Marsillac MW, Vieira RS. Assessment of artificial caries lesions through scanning electron microscopy and cross-sectional microhardness test. Indian ***J Dent Res*** 2013;24:249-54.
25. Cochrane N, Shen P, Yuan Y, Reynolds E. Ion release from calcium and fluoride containing dental varnishes. ***Aust Dent J*** 2014;59:100-5.
26. Güçlü ZA, Alaçam A, Coleman NJ. A 12-week assessment of the treatment of white spot lesions with CPP-ACP Pastes and/or fluoride varnish. ***Biomed Res Int*** 2016;15:1-9.
27. Gurunathan D, Somasundaram S, Kumar S. Casein phosphopeptide-amorphous calcium phosphate: a remineralizing agent of enamel. ***Aust Dent J*** 2012;57:404-8.
28. Choudhary P, Tandon S, Ganesh M, Mehra A. Evaluation of the remineralization potential of amorphous calcium phosphate and fluoride containing pit and fissure sealants using scanning electron microscopy. ***Indian J Dent Res*** 2012;23:157-63.

Original Article

Efficacy of Royal Jelly Extract on Inhibition of Candida Albicans Adherence on Various Types of Denture Base Material

Danai Jareemit[1], Duangporn Srisuparbh[2] and Pavinee Padipatvuthikul Didron[1]

[1]Department of General Dentistry, Faculty of Dentistry, Srinakharinwirot University, Bangkok

[2]Department of Stomatology, Faculty of Dentistry, Srinakharinwirot University, Bangkok

Abstract

Denture stomatitis is a common disease found in 60 percent of denture wearers. The causes are denture trauma and the high concentration of *Candida albicans* adherence on the inner surface of denture. Microbial adherence is the initial stage and the most important process, which causes the disease. The aim of this research was to study the efficacy of crude royal jelly extract on inhibition of *Candida albicans* adherence on various types of denture base material. The specimens of heat-cured acrylic resin, self-cured acrylic resin and tissue conditioners were placed in a various concentration of crude royal jelly extract solution, using Sabouraud Dextrose Broth as a negative control group and Nystatin as a positive control group. The standard cell suspension was added in each well and incubated at 37ºC for 24 hours. The adherence of *Candida albicans* was determined using MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and SEM (scanning electron microscopy). Adherence of *Candida albicans* was found on both heat-cured and self-cured denture base acrylic for the negative control group, but was less found on both types of tissue conditioners. In addition, crude royal jelly extract solution at a concentration of 50 mg/mL and 25 mg/mL could significantly inhibited the adherence of *Candida albicans* when compare with the negative controls group ($P < 0.05$). The increase of royal jelly concentration further reduced the adherence of *Candida albicans* on both types of denture acrylic, which was consistent with the SEM result. There was no statistical significance ($P > 0.05$) between the type of acrylic resin and the adherence of *Candida albicans.* The results obtained from this research can be used as a baseline information for further development of royal jelly products as an antimicrobial agent especially for those who wear denture.

Keywords: Acrylic resin, *Candida albicans*, Royal jelly, Tissue conditioner

Received Date: APR 27, 2017 **Accepted Date:** JUL 17, 2017

doi: 10.14456/jdat.2017.17

Correspondence to:

Danai Jareemit. Post-graduate student, Master of Science in General Dentistry, Srinakharinwirot University 114 Sukhumvit 23, Wattana District, Bangkok 10110 Thailand. Tel: 0982631026 E-mail: jareemitdanai@hotmail.com

Introduction

Denture stomatitis is one of the most common diseases in denture wearers, affecting 60 % of the population.[1-5] Denture stomatitis is caused by poor-fitting denture, improper denture border extension, and improper denture cleansing which could lead to microbial adherence and colonization.[6-9] *C. albicans* is often found as the cause of denture stomatitis.[10-12] The adherence of *C. albicans* on the inner surface of denture is the initial stage and the most important process, which causes the disease.[13] However, adherence processes are different depending on the type of denture base material, for example; heat-cured acrylic resin, self-cured acrylic resin and tissue conditioner, they have varying degrees of porosity, surface free energy, hydrophobicity and roughness. Acrylic resin is currently the most widely used denture base material. Introduction of PMMA (Polymethyl methacrylate) for using as denture base material dates back to the year 1937 when Dr. Walter Wright clinically evaluated PMMA and found that it fulfilled all the requirements of an ideal denture base material.[14] Since its introduction, PMMA has been continuously used because of its favorable working characteristics, processing ease, accurate fit, stability in oral environment, good color stability, dimensional stability, superior esthetics, repairing ease and it can be used with inexpensive equipment, however, *Candida* can adhere to the inner surface of PMMA dentures.[15] Heat-cured acrylic resin utilizes heat from hot water or ultraviolet light to activate the polymerization process, while self-cured acrylic resin utilizes chemical activator such as Dimethyl-para-toluidine.[16,17] Therefore the difference between heat-cured acrylic resin and self-cured acrylic resin is the activation process that causes free radicals. However, The polymerization of self-cured acrylic is not completed when compared to heat-cured acrylic, hence some unpolymerized monomers is left after the reaction.[18] The consequence is the reduced strength and tissue irritation although self-cured acrylic resin causes less contraction which results in more dimensional accuracy. Self-cured acrylic is suitable for repairing denture base because of its convenience. It also takes much less time for denture repair and can be done in one visit in dental clinic. Tissue conditioner has been developed in order to reduce and redistribute occlusal stress especially in patients who have thin, sharp, or badly resorbed residual alveolar ridges or chronic tissue irritation from denture forces that might damage the underlying mucosal tissues.[19] The problems of tissue conditioner is the colonization of *C. albicans* on and within it. Fungal growth is known to destroy the surface properties of tissue conditioner and this may lead to irritation of the oral tissues. This is due to a combination of increased surface roughness and high concentrations of exotoxins and metabolic products produced by the fungal colonies.[20] Unfortunately, conflicting adherence results are reported on tissue conditioner. Some *in vitro* studies reported significant inhibitory effects on *C. albicans*.[21] However, some studies showed only limited antifungal properties and no significant reduction on Candida adherence.[22] *C. albicans* adhere to polymeric surfaces by Van der Waals and electrostatic forces.[5,23,24] The development of yeast biofilm on acrylic resin occurs in 3 distinct stages after colonization. The initial stage (up to 11 hours), forming of micro-colonies, the intermediated stage (12 hours to 30 hours), extracellular matrix accumulates over colonies, and the maturation stage (38 hours to 72 hours), forming of biofilm. The forming of yeast biofilm on the inner surface of denture is the initial stage which causes the denture stomatitis.[13] Thus the prevention of *C. albicans* adherence to acrylic resin could be a possible method for prevention of denture stomatitis.[25]

In general, denture stomatitis often be treated by application of topical antifungal drug, Nystatin which is commonly used for the treatment of local fungal infection is therefore often used for treating this disease. The mechanism of Nystatin starts when forming

complexes with the ergosterol, a major component of the fungal cell membrane. When present in sufficient concentrations, it forms pores in the membrane that lead to K+ leakage, acidification, and death of the fungus.[26] Despite aforementioned benefit, the antifungal medications are chemically synthesized and possibly lead to drug-resistance when used continuously. Nowadays, the interest in medicinal nature as a source of antimicrobial agents has grown dramatically. Recently, there were sequentially reports of the *in vitro* and *in vivo* antibacterial action of Royal jelly, which is a natural product.[27,28] It is a milky secretion produced by young worker honeybees, containing numerous compounds such as water, proteins, amino acids, minerals and vitamins. It was also found to contain 10-HDA (Trans-10-hydroxy-2-decenoic acid), which is an efficient bacteriostatic against gram-positive and gram-negative bacterias.[29] The aim of this study was to investigate the efficacy of CRJE (Crude Royal Jelly Extract) on the inhibition of *C. albicans* adherence on various types of denture base material.

Materials and methods

The sample size calculation

The sample size was calculated using G* Power 3.0 for Windows XP program.[30] The obtained number of sample in each group was eight specimens. Four experimental groups are heat-cured acrylic resin, self-cured acrylic resin, Soft-liner and Dura conditioner. Nystatin (23 mg/mL) (Tystatin Oral Suspension, T.O. Phama Co.,Ltd., Thailand) was used as a positive control and SDB (Sabouraud Dextrose Broth) (Himedia, USA) was used as the negative control.

Preparation of acrylic resin specimens

Brass metal mold was used to fabricate samples of 10 mm in diameter and 3 mm in thickness. A thin layer of Vaseline (Unilever, Thailand) was applied inside the mold as a lubricant, self-cured acrylic resin (ProBase Cold, Ivoclar-Vivadent AG, Liechtenstein) and tissue conditioners (Soft liner, GC corporation, Tokyo, Japan / Dura conditioner, Dental Mfg., Worth, IL) were prepared according to the manufacturer's recommended ratio shown in Table 1 and placed in the mold, the surface was finished with a flat mirror to obtain a flat surface. The heat-cured acrylic resin (Vertex-Dental, B.V., Netherlands) was prepare by pouring pink wax into the mold, the pink wax samples were flasked and heat-cured acrylic was packed to obtain heat-cured acrylic samples of the same dimension. The Vaseline on specimens' surface was cleaned off using dishwashing liquid (Sunlight®, Unilever, Thailand) and the specimens were soaked in distilled water for 24 hours to get rid of the residual monomer. They were then sterilized by ethylene oxide gas.

Preparation of *C. albicans*

The *Candida* strains used in this study was *C. albicans* (ATCC 90028), which cultured on SDA (Sabouraud Dextrose Agar) (Himedia, USA) by incubation at 37°C for 24 hours. Then the colonies were grown in SDB and incubated at 37°C for 24 hours and the cell suspension was adjusted to 0.5 McFarland ($1x10^6$ CFU (Colony forming unit))

Preparation of royal jelly extract

Royal jelly powder (Su Pha Bee Farm, Chiang Mai, Thailand) was extracted with 20 percent ethanol at a concentration of initial solution royal jelly equals 100 mg/mL. The supernatant was collected after centrifugation (TOMY® MX-160, American Laboratory Trading, USA) at a temperature of 4°C, 10,000 rpm for 10 minutes and freeze dried (FreeZone 2.5, LABCONCO., USA). Then CRJE (Crude Royal Jelly Extract) was weighed and dissolved in SDB for the initial concentration of 100 mg/mL. The clear solution was sterilized through a membrane filter paper with a pore size of 0.20 micrometers. (Minisart®, Sigma-Aldrich Pte Ltd., Singapore)

Table 1 *Samples of two acrylic resins and two tissue conditioners*

Products	Manufacturers	Polymerization Method	Composition
Heat-cured acrylic resin			
Vertex Rapid Simplified Powder Lot.XR135P03 Liquid Lot.XR15L01	Vertex-Dental B.V. Netherlands	Heat-cured	**Powder** Polymethyl methacrylate, Accelerator, Color agents **Liquid** Methyl methacrylate, Cross linker, Accelerator
Self-cured acrylic resin			
ProBase Cold Pink NO.5 Powder Lot. R82188 Liquid Lot. S03282	Ivoclar-Vivadent AG Liechtensten	Self-cured	**Powder** Polymethyl methacrylate, Softening agent, Benzoyl peroxide, Catalyst, Pigments **Liquid** Methyl methacrylate, Dimethacrylate, Catalyst
Tissue conditioners			
1.Soft-liner (Soft denture reline material) Powder Lot.1406101 Liquid Lot.1406052	GC corporation Tokyo, Japan	Self-cured	**Powder** Polymethyl methacrylate **Liquid** Butylphthalyl butylglycolate, Ethanol
2.Dura conditioner (Reliance) Powder Lot.022305 Liquid Lot.062309	Dental Mfg.Co. Worth, IL	Self-cured	**Powder** Polymethyl methacrylate **Liquid** 2-Ethylhexyl diphenyl phosphate, Bis(2-Ethylhexyl) phenyl phosphate, Triphenyl phosphate

MIC (Minimum Inhibitory Concentration) and MFC (Minimum Fungicidal Concentration)

The sterile CRJE solution was diluted by Two-fold dilution at a concentration of 100 mg/mL, 50 mg/mL, 25 mg/mL, 12.5 mg/mL, 6.25 mg/mL and 3.125 mg/mL respectively; at the volume of 1 mL. *C. albicans* suspension 1 mL was then added. So, a final concentration of CRJE in the treatment group was 50 mg/mL, 25 mg/mL. 12.5 mg/mL, 6.25 mg/mL, 3.125 mg/mL, 1.0625 mg/mL, respectively. A negative control group was used SDB 2 mL. These tubes were incubated at 37°C temperature for 24 hours. After that, the yeast colonies were observed to get the MIC and MFC value.

Adhesion assay and analysis

The candida adherence to the acrylic resin specimens was assayed in Broth dilution method and MTT assay. Each specimen was placed in a well containing 500 µl of CRJE at a concentration of 12.5 mg/mL, 25 mg/mL and 50 mg/mL, SDB used as negative control group and Nystatin of concentration 23 mg/mL used as

positive control group. The 500 µl of the standard cell suspension was then added in each well and incubated at 37°C for 24 hours to allow the cells to attach to the surface of the specimens.[31] After the incubation, the specimens were washed in a standard manner by dipping in sterile PBS (Phosphate Buffered Saline) to remove loosely attached cells, then placed in a new 24-well plates with a 600 µl volume of SDB and a 150 µl volume of MTT Stock. Shake and then incubated at 37°C for 4 hours to find the Formazan purple crystals stuck on the specimens. The specimens were placed in a new 24-well plates with a 700 µl volume of DMSO (Dimethyl sulfoxide) to dissolve the crystals and then into the shaker (Rocker-Shaker®, Biosan, Latvia) for 15 minutes. It has a purple solution, were determined in terms of optical density at a wavelength of 570 nm using DMSO solution as a blank to analyze.

Scanning electron microscopy for *C. albicans*-attached specimens

C. albicans were adhered to the acrylic resin as described above and were incubated at 37°C for 24 hours, specimen were washed with PBS and then soaked in 2.5 percent Glutaralaldehyde in PBS for two hours, 25°C, then washed with PBS, dehydrated with Alcohol and sputter coated with platinum for investigation with scanning electron microscopy (JEOL, USA) at a magnification of 400 times.

Statistical analysis

The adherence of *C. albicans* on acrylic resin surfaces were analyzed using one-way and two-way ANOVA (analysis of variance). The analysis was done with a statistic package for social science (SPSS for Windows® version 22). For all of the statistic analysis, a P-value below 0.05 was considered statistically significant.

Results

C. albicans adherence was investigated in four types of denture base materials. The results were statistically analyzed using one-way ANOVA and Welch test. It was found that the groups of denture base material were significantly different on the *C. albicans* adherence. Subsequent Post-hoc test revealed that both types of acrylic resin had more *C. albicans* adherence while both of tissue conditioners had less *C. albicans* adherence as shown in Table 2. We therefore decided to continue the study on the efficacy of various concentration of CRJE on inhibition of *C. albicans* adherence on both acrylic resin materials.

Table 2 *Adherence of C. albicans in SDB to different types of denture base material*

Denture materials	OD_{570} (Mean ± SD) (n=8)
Heat-cured acrylic resin	0.4155 ± 0.0996[a]
Self-cured acrylic resin	0.4289 ± 0.1191[a]
Dura conditioner	0.1255 ± 0.0103[b]
Soft-liner	0.1196 ± 0.0090[b]

The fungal inhibition and fungicidal effect of CRJE against *C. albicans* can be expressed in MIC and MFC values, which were 12.5 mg/mL and 50 mg/mL respectively. Therefore, the concentration of CRJE which used in this study were 12.5, 25 and 50 mg/mL. To determine whether the correlation between the types of cured acrylic and concentration of CRJE solution affect the adherence of *C. albicans*, two-way ANOVA and Levene's Test were used. Tests of Between-Subjects Effects showed no correlation between the type of

acrylic resin and the concentration of CRJE solution (P = 0.993). Type of acrylic resin did not significantly affected the adherence of *C. albicans* ($P > 0.05$). In contrast, the concentration of CRJE significantly affected the adherence of *C. albicans* ($P < 0.05$).

The adherence of *C. albicans* to the acrylic resin, following a 24-hour exposure to various sublethal concentrations of CRJE are presented in Table 3 which shows CRJE at a concentration of 50 mg/mL, 25 mg/mL and positive control group could significantly inhibit the adherence of *C. albicans* when compare with the negative controls group ($P < 0.05$). The percent reduction in the adherence of *C. albicans* are presented in Table 4, which showed the percent reduction of *C. albicans* of CRJE at a concentration of 50 mg/mL, 25 mg/mL and 12.5 mg/mL and a positive control group compared to negative controls group on both of acrylic resins type. As mentioned above, we found that CRJE at a concentration of 50 mg/mL can inhibit the adherence of *C. albicans* up to half when compared with the negative control group and CRJE at a concentration between 25-50 mg/mL have 46.15-52.03 percent reduction compared to Nystatin 23 mg/mL which has 53.37-54.01 percent reduction. This reduction was concentration-dependent, since higher concentrations resulted in higher blockage of adherence on both of acrylic resin type as shown in Figure 1. This result was consistent with the SEM result. We found the adherence of *C. albicans* on specimens in CRJE at 3 concentrations of 50 mg/mL, 25 mg/mL, 12.5 mg/mL and positive control group were decreased compared to the negative control group. When the concentration of CRJE increased, the adherence of *C. albicans* reduced for both of acrylic resins type as shown in scanning electron micrographs (Fig. 2).

Table 3 *Adherence of C. albicans to denture acrylic after exposure to 12.5 mg/mL, 25 mg/mL, 50 mg/mL, Nystatin 23 mg/mL and SDB when comparing between Heat-cured acrylic resin, Self-cured acrylic resin.*

	OD_{570} (Mean ± SD) (n=8)	
	Heat-cured acrylic resin	**Self-cured acrylic resin**
CRJE 50 mg/mL	0.2021 ± 0.0250*	0.2058 ± 0.0124*
CRJE 25 mg/mL	0.2238 ± 0.0491*	0.2143 ± 0.0117*
CRJE 12.5 mg/mL	0.2821 ± 0.0396	0.2881 ± 0.1022
Nystatin 23 mg/mL	0.1938 ± 0.0271*	0.1973 ± 0.0204*
SDB	0.4155 ± 0.0996	0.4289 ± 0.1191

** P < 0.05 significantly differences compare, CRJE: Crude Royal Jelly Extract, Nystatin: positive control group, (SDB) Sabouraud Dextrose Broth: negative control group*

Table 4 *Percent reduction of C. albicans adherence in CRJE at a concentration of 12.5 mg/mL, 25 mg/mL, 50 mg/mL, Nystatin 23 mg/mL and SDB when comparing between Heat-cured acrylic resin and Self-cured acrylic resin*

	Reduction in adherence (%)	
	Heat-cured acrylic resin	**Self-cured acrylic resin**
CRJE 50 mg/mL	51.36	52.03
CRJE 25 mg/mL	46.15	50.04
CRJE 12.5 mg/mL	32.10	32.82
Nystatin 23 mg/mL	53.37	54.01
SDB	0	0

CRJE: Crude Royal Jelly Extract, Nystatin: positive control group, SDB (Sabouraud Dextrose Broth): negative control group.

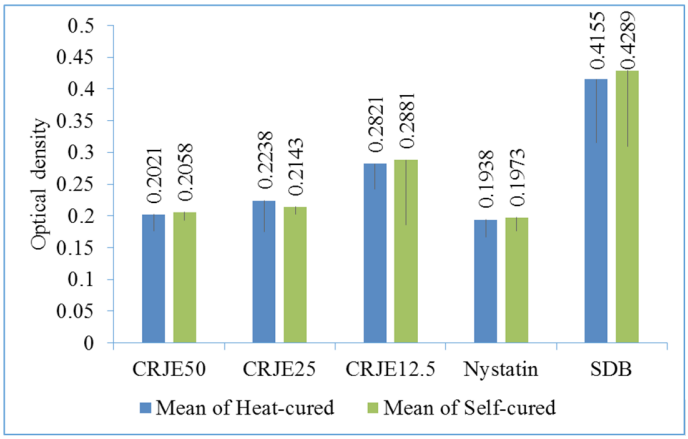

Figure 1 *Mean of the absorbance of CRJE at a concentration of 50 mg/mL (CRJE50), 25 mg/mL (CRJE25), 12.5 mg/mL (CRJE12.5), Nystatin 23 mg/mL (Positive) and SDB (Negative) of heat-cured acrylic resin (H) and self-cured acrylic resin (S).*

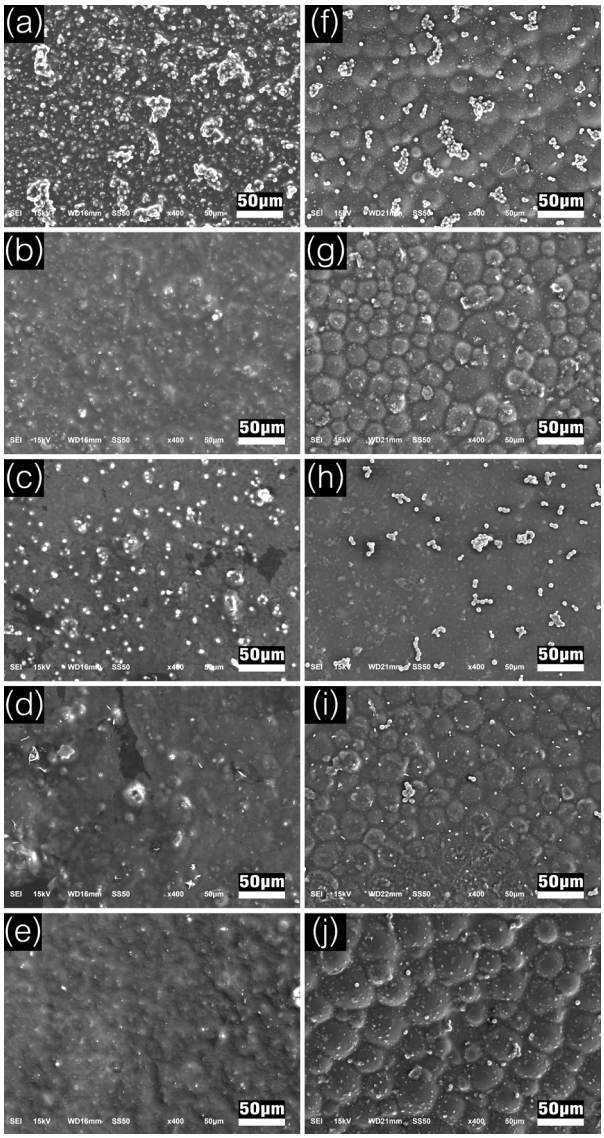

Figure 2 *Scanning electron micrographs at 400x magnification of C. albicans adherence, a comparison between heat-cured acrylic resin after exposure to SDB (a), Nystatin 23 mg/mL (b), CRJE 12.5 mg/mL (c), CRJE 25 mg/mL (d) CRJE 50 mg/mL (e) and self -cured acrylic resin after exposure to SDB (f), Nystatin 23 mg/mL (g), CRJE 12.5 mg/mL (h), CRJE 25 mg/mL (i), CRJE 50 mg/mL (j)*

Discussion

The objective of this study was to study the efficacy of CRJE on inhibition of *C. albicans* adherence on various types of denture base materials as a possible method to treat and prevent denture stomatitis in denture wearers. The microbial adhesion on denture base materials varies, depend on the type of material. This could impact the physical properties such as porosity, surface free energy, hydrophobicity, or roughness.[3,5,22,23] *Candida* can adhere to the inner surface of dentures made of PMMA because it is a hydrophobic material. In addition, epithelial cell can bind easily with the hydrophobic surfaces. Microbial adherence arises from hydrophobic interaction and Lewis acid-base interaction. If the surface is very hydrophobic (low surface energy), microbial adherence is increased.[15] Regarding the study on hydrophobicity, Minagi *et al.*, concluded that there was a higher adherence of micro-organisms to the material which had a surface free-energy closest to that of the specific organism and that hydrophobic interaction is significantly important in the initial attachment of yeasts to polymeric surfaces.[32] It was found that when the culture was in a state without any treatment, both types of acrylic resins had more *C. albicans* adherence and both of tissue conditioners had less *C. albicans* adherence. Probably because of the material components of tissue conditioners react with the microorganisms in the mouth or Plasticizer, such as Benzyl benzoate and Benzyl salicylate is effective in killing fungus.[31,33,34] Unlike the results from previous study done by Hema *et al.*, in 2011, they did not find that tissue conditioners (Viscogel and GC soft) can inhibit the adherence of *C. albicans*.[35]

In term of the efficacy of CRJE on inhibition of *C. albicans* (Ayse Nedret Koc *et al.,* 2011), they evaluated the ability of honeybee products including royal jelly to inhibit the growth of 40 yeast strains of *C. albicans*, *C. glabrata*, *C. krusei*, and *Trichosporon spp*. Using the broth microdilution method, minimal inhibitory concentration ranges 0.06-1 µg/mL had antifungal activities.[36]

The results of the study are consistent with the hypothesis that CRJE can inhibit the growth of *C. albicans* and adherence of *C. albicans* on both type of acrylic resins. CRJE at a concentration between 25-50 mg/mL can significantly inhibit the adherence of *C. albicans*, this is approximately equivalent to Nystatin 23 mg/mL thus it could be an alternative anti-fungal product to Nystatin. The advantage of a CRJE such as, unlike synthetic drugs, the royal jelly is a natural product that cause less allergic reaction and contains no chemicals that are harmful to humans. Also the CRJE processing does not cause result an environmental pollution.

This research result is similar to Moselhy *et al.* which conducted research on the inhibition of microbial, using royal jelly from Egypt and China in experiments done by disc diffusion method. The results showed effective inhibition against bacteria. It also has anti-fungal effect includes *Aspergillus fumigant, Aspergillus niger, C. albicans* and a *Syncephalastrum racemosum*. The best concentration of royal jelly to inhibit *C. albicans* is 15 mg/mL,[37] in which this study found that when the concentration is higher, there was also a much wider zone of inhibition. However we noticed that the concentration of the royal jelly extract from many studies that can inhibit the growth of pathogenic *C. albicans* were found to be different. A study of Bachnova *et al.* in 2004, which explained the efficacy of royal jelly solution cannot be compared with the results of other studies because of many factors including microorganisms of different species and the different environment and culture. The different bee species in each country were different. Several studies have revealed specific components of RJE, including 10-HDA, Royalisin and Jelleines, are the main antimicrobial biaoctives, they have a significant antibacterial potential. A study of RJE was conducted by Takenaka *et al.* in 1986, described the antibacterial and antifungal effects of 10-HDA against *Bacillus subtilis, Staphylococcus aureus,* and *Escherichia coli.*[39] One of

the studies that evaluated the antibacterial activity of Royalisin has been reported against *B. subtilis.* This inhibition was equal to that of tetracycline at 50 µg/mL.[40] Jelleines are small peptides, which have antimicrobial properties against several gram-positive cocci (*S. aureus, S. saprophyticus,* and *B. subtilis*) and gram-negative rods (*E. coli, E. cloacae, K. pneumoniae, and P. aeruginosa*), as well as yeast (*C. Albicans*).[41]

A study on the ability of other natural extracts that inhibit the adherence of *C. albicans* by Taweechaisupapong *et al.* in 2006, they studied an Inhibitory effect of Streblus asper leaf-extract on adherence of *C. albicans* to denture acrylic, using various sublethal concentrations of Streblus asper leaf ethanolic extract. The experiments were performed on self-cured acrylic resin by Broth dilution method, combined with A colorimetric tetrazolium assay using XTT ((2, 3)-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-(12)-2H-tetrazolium hydroxide). The results showed that the Streblus asper leaf-extract concentration of 31.2 mg/mL, 62.5 mg/mL and 125 mg/mL, led to a significant reduction ($P < 0.05$) of *C. albicans* compared to the control group. The reduction was concentration-dependent, since higher concentrations resulted in higher blockage of adherence. The Streblus asper leaf-extract could affect the cell wall of fungi, such as creating extracellular components and chemical surface adherence inhibiting effect.[42]

In a study using synthetic substances to inhibit the adherence of *C. albicans* on various surfaces. Lin Zhou *et al.* used Parylene® to inhibit the adherence of *C. albicans* on the surface of denture made of heat-cured acrylic resin. The result showed that Parylene® had the ability to reduce the adherence of pathogenic *C. albicans* on the acrylic resin. Cell counts and XTT assay also showed significant reduction.[43]

When comparing properties and surface characteristics of heat-cured acrylic resin and self-cured acrylic resin, we found that heat-cured acrylic resin has less surface roughness than self-cured acrylic resin which is consistent with the less adherence of *C. albicans* on heat-cured resin.[44] However, the results from this study found no difference in the adherence of *C. albicans* in both of acrylic resins type. This is probably because of the specimens preparation process

This research is only an in vitro study, therefore the results from this study may differ from results from an experiment conducted in oral condition. This is an important factor encouraging the growth of fungus. Thus more studies are still needed to provide more information regarding the mechanism of the adherence of *C. albicans.* In addition, a study on CRJE pre-coating application to the denture base material could also be done instead of soaking application.

Conclusion

The study concluded that CRJE has the ability to inhibit the adherence of *C. albicans* on the surface of both heat-cured acrylic resin and self-cured acrylic resin. This is an alternative development the product to patients who wear dentures which made from acrylic resin. Especially the elderly or patients with restrictions on the hand movement.

Acknowledgments

This work was supported by a research grant from the Faculty of Dentistry, Srinakrinwirot University. Authors would also like to thank Dr. Waiyawut Yoonisil for his advices on the Statistical analysis.

References

1. He XY, Meurman JH, Kari K, Rautemaa R, Samaranayake LP. *In vitro* adhesion of Candida species to denture base materials. ***Mycoses*** 2006;49:80-4.

2. Jain D, Shakya P. An *in vitro* study on effect of Delmopinol application on Candida albicans adherence on heat cured denture base acrylic resin: a thorough study. ***Indian J Dent Res*** 2013;24:645.

3. Uzunoglu E, Yildirim Bicer AZ, Dolapci I, Dogan A. Biofilm-forming ability and adherence to poly-(methyl-methacrylate) acrylic resin materials of oral *Candida albicans* strains isolated from HIV positive subjects. ***J Adv Prosthodont*** 2014;6:30-4.

4. Nawasrah A, AlNimr A, Ali AA. Antifungal Effect of Henna against *Candida albicans* Adhered to Acrylic Resin as a Possible Method for Prevention of Denture Stomatitis. ***Int J Environ Res Public Health*** 2016;13.

5. Gebremedhin S, Dorocka-Bobkowska B, Prylinski M, Konopka K, Duzgunes N. Miconazole activity against *Candida* biofilms developed on acrylic discs. ***J Physiol Pharmacol*** 2014;65:593-600.

6. Waters MG, Williams DW, Jagger RG, Lewis MA. Adherence of *Candida albicans* to experimental denture soft lining materials. ***J Prosthet Dent*** 1997;77:306-12.

7. Hoshi N, Mori H, Taguchi H, Taniguchi M, Aoki H, Sawada T, *et al.* Management of oral candidiasis in denture wearers. ***J Prosthodont Res*** 2011;55:48-52.

8. Iseri U, Uludamar A, Ozkan YK. Effectiveness of different cleaning agents on the adherence of *Candida albicans* to acrylic denture base resin. ***Gerodontology*** 2011;28: 271-6.

9. Skupien JA, Valentini F, Boscato N, Pereira-Cenci T. Prevention and treatment of *Candida* colonization on denture liners: a systematic review. ***J Prosthet Dent*** 2013;110:356-62.

10. Pereira-Cenci T, Del Bel Cury AA, Crielaard W, Ten Cate JM. Development of Candida-associated denture stomatitis: new insights. ***J Appl Oral Sci*** 2008;16:86-94.

11. Kanathila H, Bhat AM, Krishna PD. The effectiveness of magnesium oxide combined with tissue conditioners in inhibiting the growth of *Candida albicans*: An *in vitro* study. Indian ***J Dent Res*** 2011;22:610.

12. Kang SH, Lee HJ, Hong SH, Kim KH, Kwon TY. Influence of surface characteristics on the adhesion of *Candida albicans* to various denture lining materials. ***Acta Odontol Scand*** 2013;71:241-8.

13. Tari BF, Nalbant D, Dogruman Al F, Kustimur S. Surface roughness and adherence of *Candida albicans* on soft lining materials as influenced by accelerated aging. ***J Contemp Dent Pract*** 2007;8:18-25.

14. Peyton FA. History of resins in dentistry. ***Dent Clin North Am*** 1975;19:211-22.

15. Azuma A, Akiba N, Minakuchi S. Hydrophilic surface modification of acrylic denture base material by silica coating and its influence on *Candida albicans* adherence. ***J Med Dent Sci*** 2012;59:1-7.

16. Winkler S, Wood R, Facchiano AM, Boberick KG, Patel AR. Prosthodontic Self-treatment with Acrylic Resin Super Glue: A Case Report. ***J Oral Implantol*** 2006;32:132-6.

17. Rashid H, Sheikh Z, Vohra F. Allergic effects of the residual monomer used in denture base acrylic resins. ***Eur J Dent*** 2015;9:614-9.

18. Chaves CA, Machado AL, Vergani CE, de Souza RF, Giampaolo ET. Cytotoxicity of denture base and hard chairside reline materials: a systematic review. ***J Prosthet Dent*** 2012;107:114-27.

19. McCabe JF. A polyvinylsiloxane denture soft lining material. ***J Dent*** 1998;26:521-6.

20. Masella RP, Dolan CT, Laney WR. The prevention of the growth of *Candida* on silastic 390 soft liner for dentures. ***J Prosthet Dent*** 1975;33:250-7.

21. Wright PS. The effect of soft lining materials on the growth of *Candida albicans*. ***J Dent*** 1980;8:144-51.

22. Pereira-Cenci T, Cury AA, Cenci MS, Rodrigues-Garcia RC. *In vitro* Candida colonization on acrylic resins and denture liners: influence of surface free energy, roughness, saliva, and adhering bacteria. ***Int J Prosthodont*** 2007; 20:308-10.

23. Oncul B, Karakis D. The effect of two artificial salivas on the adhesion of *Candida albicans* to heat-polymerized acrylic resin. ***J Adv Prosthodont*** 2015;7:93-7.

24. Akalin-Evren B, Kulak-Ozkan Y, Ozcan M, Kadir T. *Candida albicans* adhesion on reinforced polymethyl-methacrylate denture resin: effect of fibre architecture and exposure to saliva. ***Gerodontology*** 2014;31:194-201.

25. Chandra J, Kuhn DM, Mukherjee PK, Hoyer LL, McCormick T, Ghannoum MA. Biofilm formation by the fungal pathogen Candida albicans: development, architecture, and drug

resistance. ***J Bacteriol*** 2001;183:5385-94.
26. Ellepola AN, Samaranayake LP. The effect of limited exposure to antifungal agents on the germ tube formation of oral Candida albicans. ***J Oral Pathol Med*** 1998;27:213-9.
27. Bilikova K, Huang SC, Lin IP, Simuth J, Peng CC. Structure and antimicrobial activity relationship of royalisin, an antimicrobial peptide from royal jelly of Apis mellifera. ***Peptides*** 2015;68:190-6.
28. Bilikova K, Kristof Krakova T, Yamaguchi K, Yamaguchi Y. Major royal jelly proteins as markers of authenticity and quality of honey. ***Arh Hig Rada Toksikol*** 2015;66: 259-67.
29. Fujii A, Kobayashi S, Kuboyama N, Furukawa Y, Kaneko Y, Ishihama S, *et al.* Augmentation of wound healing by royal jelly (RJ) in streptozotocin-diabetic rats. ***Jpn J Pharmacol*** 1990;53:331-7.
30. Faul F, Erdfelder E, Lang AG, Buchner A. G*Power 3: A flexible statistical power analysis program for the social, behavioral, and biomedical sciences. ***Behav Res Methods*** 2007;39:175-191.
31. Gautam R, Singh RD, Sharma VP, Siddhartha R, Chand P, Kumar R. Biocompatibility of polymethylmethacrylate resins used in dentistry. ***J Biomed Mater Res B Appl Biomater*** 2012;100:1444-50.
32. Minagi S, Miyake Y, Inagaki K, Tsuru H, Suginaka H. Hydrophobic interaction in *Candida albicans* and Candida tropicalis adherence to various denture base resin materials. ***Infect Immun*** 1985;47:11-14.
33. Bulad K, Taylor RL, Verran J, McCord JF. Colonization and penetration of denture soft lining materials by *Candida albicans*. ***Dent Mater*** 2004;20:167-75.
34. Serrano-Granger C, Cerero-Lapiedra R, Campo-Trapero J, Del Rio-Highsmith J. *In vitro* study of the adherence of *Candida albicans* to acrylic resins: relationship to surface energy. ***Int J Prosthodont*** 2005;18:392-8.
35. Kanathila H, Bhat AM, Krishna PD. The effectiveness of magnesium oxide combined with tissue conditioners in inhibiting the growth of *Candida albicans*: an *in vitro* study. ***Indian J Dent Res*** 2011;22:613.
36. Koc AN, Silici S, Kasap F, Hormet-Oz HT, Mavus-Buldu H, Ercal BD. Antifungal activity of the honeybee products against *Candida* spp. and *Trichosporon* spp. ***J Med Food*** 2011;14:128-34.
37. Moselhy A FA, Kamel A. An evaluation of the potent antimicrobial matter analysis of the royal jelly. ***Life Sci*** 2013;10.
38. Bachanová K, Klaudiny J, Kopernicky J, Simúth J. Identification of honeybee peptide active against Paenibacillus larvae larvae through bacterial growth-inhibition assay on polyacrylamide gel. ***Apidologie*** 2002;33:259-69.
39. Takenaka T, Yatsunami K, Echigo T. Changes in Quality of Royal Jelly during Storage. ***Nippon Shokuhin Kogyo Gakk*** 1986;33:1-7.
40. Bilikova K, Wu GS, Simuth J. Isolation of a peptide fraction from honeybee royal jelly as a potential antifoulbrood factor. ***Apidologie*** 2001;32: 275-83.
41. Fontana R, Mendes MA, de Souza BM, Konno K, César LM, Malaspina O, *et al.* Jelleines. A family of antimicrobial peptides from the Royal Jelly of honeybees (Apis mellifera). ***Peptides*** 2004;25:919-28.
42. Taweechaisupapong S, Klanrit P, Singhara S, Pitiphat W, Wongkham S. Inhibitory effect of Streblus asper leaf-extract on adhesion of Candida albicans to denture acrylic. ***J Ethnopharmacol*** 2006;106:414-7.
43. Zhou L, Tong Z, Wu G, Feng Z, Bai S, Dong Y, *et al.* Parylene coating hinders *Candida albicans* adhesion to silicone elastomers and denture bases resin. ***Arch Oral Biol*** 2010;55:401-9.
44. He XY, Meurman JH, Kari K, Rautemaa R, Samaranayake LP. In vitro adhesion of *Candida* species to denture base materials. ***Mycoses*** 2006;49:80-4.

Original Article

A Preliminary Study to Compare the Adaptability and Nanoleakage of Resin-Based Materials at the Cervical Dentin of Class II Cavity

Sukamon Kochotwuttinont[1], Mayurach Pipatphatsakorn[1] and Kornchanok Wayakanon[1]

[1]Department of Restorative Dentistry, Naresuan University, Phitsanulok

Abstract

Resin composite is the most popular tooth-colored material for the dental restoration. The polymerization shrinkage is an unavoidable disadvantage of this material which is associated with the gap formation and the secondary caries. Bonding system is used to eliminate these problems. However, the gingival margin of the proximal cavity remains the most common area found the restorative defects. An open sandwich technique has been suggested to address this problem. The aim of this study is to evaluate the degree of nanoleakage and adaptability of different lining materials in open sandwich technique. The slot cavities were prepared on the proximal surface of teeth with the gingival margin 1 mm below the CEJ. The teeth were divided into 5 groups (n=5): group I a flowable resin composite, group II a bulk fill flowable resin composite, group III a resin modified glass ionomer cement, group IV and V no lining material. Samples in the group I-III and V were restored with nanofilled resin composite while group IV were restored with bulk fill resin composite. All groups were thermocycled, processed with silver nitrate solution and observed under SEM. The silver nitrate deposited entire thickness of hybrid layer, in the dentinal tubules and on the resin tags in group I, II, IV and V. In group III, it deposited within the modified hybrid layer. The silver nitrate deposition was highest in group V (70.44 %) while the group II (55.29 %) was the lowest. The gap formation was found in almost all outer 1/3 of samples. The width of gap was different among materials. In the conclusion, the type of lining materials had an influence on the degree of adaptability to dentin while it did not effect on nanoleakage of bonding system. The bulk fill resin composite could improve the adaptability of the restoration to cervical dentin margin.

Keywords: Adaptability, Nanoleakage, Bulk fill resin composite, Open sandwich technique, Cervical dentin

Received Date: APR 27, 2017 **Accepted Date:** JUL 17, 2017

doi: 10.14456/jdat.2017.18

Correspondence to:

Kornchanok Wayakanon. 99 M. 9 Tha Pho, Muang, Phitsanulok 65000 Thailand Department of Restorative Dentistry, Naresuan University, Phitsanulok, Thailand Tel: 094-336-4994 E-mail: wkornchanok@hotmail.com

Introduction

A resin composite material becomes more popular as a filling material for the proximal cavities of the posterior teeth. It requires an uncomplicated form of the cavity preparation along with the preservation of tooth structure. However, the undeniable problems of light cured resin composite is the polymerization shrinkage. It is associated with the shrinkage stress consequently the occurrence of the gap formation, the post-operative sensitivity, secondary caries and the bond failure.[1,2] Many studies reported marginal leakage at the cervical dentin of proximal cavities.[3,4] The moisture contamination and the incomplete light curing at the bottom of the proximal cavities influence on this defect. The stiffness of the resin composites may not establish the proper adaptation to the internal surfaces or cavosurface of the proximal cavities.[5] Applying the adhesive system on dentin is challenged due to the difference components in the natural structure of dentin.[4] The different techniques have been suggested to reduce polymerization shrinkage and improve the adaptability of resin composite restorations such as the technique of light curing, placing of materials into cavities or applying other materials along with resin composite namely the open sandwich technique. For the latter technique, the materials with low modulus of elasticity or low polymerization shrinkage such as the flowable resin composite and resin modified glass ionomer cement (RMGIC) are advocated as a the gingival liner or intermediate layer.[6-12]

RMGICs have a chemical adhesion to the dentin and an anti-cariogenic effect. When applying RMGIC as the gingival lining materials along with the resin composite restoration, the total volume of the resin composite decreases resulting in the reduction of the shrinkage stress within the resin composite materials.[13] Some studies found the substantial improvement of the marginal adaptation of the filling materials at the gingival margins.[6,7] However, some studies showed the gaps at the RMGIC-dentin interface.[14]

An elastic cavity wall concept has been presented by using the flowable resin composite as a lining material. Due to the low modulus of elasticity, this gingival liner functions as a stress absorbing layer and reduces shrinkage stress at resin-dentin interface.[8] Various studies reported that the placement of the flowable resin composite as the gingival liner minimizes the leakage at the gingival floor[9-11] and reduces the gap formation at the internal margins.[12] However some studies did not find any advantages from flowable resin composite because of the low filler content and high polymerization shrinkage.[15,16]

Bulk fill resin composites have been recently developed to facilitate the clinical procedure. This material has the modification of the fillers by either reducing the filler content or increasing the filler-particle size in order to reduce the light scatter at the filler-matrix interface and increase the degree of light transmission.[17-20] They are subsequently able to fill into the cavity with the thickness 4-5 mm. Some studies reported that the bulk fill resin composites had less polymerization shrinkage stress and better marginal adaptation.[17,21] This material has been also suggested to use as an alternative gingival lining material.

A nanoleakage investigation is a common method to investigate the quality of an adhesive system. It had been shown as nanometer-sized spaces within a hybrid layer even there was a gap-free gingival margin. A silver nitrate is the most popular substance to be used to detect the nano-spaces by observing its deposition under a high magnification Scanning Electron Microscope (SEM).[22,23] This leakage is the result of an incomplete polymerization and infiltration of adhesive resin including the contamination at the bonding area. The nanoleakage is the considerable pathway for the penetration of bacterial products, oral fluid and dentinal fluid related to a hydrolytic degradation of adhesive resin and the bond failure.[22,24]

Although using the lining materials in the class II resin composite open sandwich techniques improve

the marginal adaptability, there are some studies reported nanoleakage at the cervical dentin.[25] Both the marginal adaptability and the nanoleakage influence on the quality of the resin composite restorations. Nevertheless, there have been few studies to evaluate both adaptability and degree of nanoleakage of different liner materials in the class II resin composite open sandwich technique at the cervical dentin margin. Since the limitation of the information, the aims of this study were 1) to evaluate a degree of nanoleakage at a cervical dentin of three lining materials and high viscosity bulk fill resin composite in class II open sandwich technique and 2) to evaluate adaptability to a cervical dentin of three liner materials and high viscosity bulk fill resin composite in class II open sandwich technique.

Materials and Methods

1. Sample preparation

This study was approved by the Ethics committee of Naresuan University (IRB No. 578/59). The maxillary premolar teeth of patients aged above 20 years old recently extracted for orthodontic reasons were collected. They must have the normal morphological feature, no cavity, no restorations and no crack line or craze line. The extracted teeth were collected in 10 % formalin solution no longer than one month. Calculus and soft tissue were removed. All teeth were then submerged in fresh 10 % formalin solution for 2 weeks and stored in 0.1 % Thymol solution at room temperature.[26-29]

All teeth were mounted with sticky wax in the silicone blocks. After that occluso-distal slot cavities were prepared with 4-mm width in bucco-lingual direction. The gingival wall was finished 1 mm cervically to the CEJ to keep gingival margin on dentin. The width of gingival wall was 1.5 mm in the mesio-distal direction by high speed fissure diamond burs (#835 FG 016 Jota, Switzerland). Each bur was replaced with a new one after five cavity preparations. After that all prepared teeth were horizontally sectioned on the occlusal surface by low speed diamond saw device with water coolant to receive the tooth samples with 4 mm occluso-cervical height. Then a tofflemire matrix holder and a metal band were placed. The teeth were assigned into 5 groups (N=24).

Group I (FC): Conventional flowable resin composite and nanofilled resin composite;
Filtek Z350 XT flowable resin®, 3M ESPE, n=5
Filtek™ Z350XT Universal Restorative, 3M ESPE

Group II (BF): Bulk fill flowable resin composite and nanofilled resin composite;
SureFil SDR Flow®, Dentsply Caulk, n=5
Filtek™ Z350XT Universal Restorative, 3M ESPE

Group III (GI): Resin-modified glass ionomer cement and nanofilled resin composite;
Fuji II LC capsule®, Accord, n=4
Filtek™ Z350XT Universal Restorative, 3M ESPE

Group IV (BFCo): No liner material, high viscosity bulk fill resin composite;
Filtek™ Bulk Fill Posterior Restorative, 3M ESPE, n=5

Group V (Co): No liner material, conventional nanofilled resin composite;
Filtek™ Z350XT Universal Restorative, 3M ESPE, n=5

All cavities in group I and II were etched with 37 % phosphoric acid (Scotchbond™Etching liquid, 3M ESPE) for 15s then rinsed with water jet for 30s and gently air dried for 30 seconds. The bonding agent (Adper™Single Bond2, 3M ESPE) was applied according to the manufacturer's instruction. For the group III, the GC conditioner liquid was applied to gingival floor for 10s then rinsed with water jet for 30s and gently dried for 30 seconds. The thickness of lining materials in group I, II and III was 1 mm. It is considered and adjusted with the periodontal probe by measuring the occlusally remaining space before light curing for 20s.

After placement the lining materials, the cavities (group I, II and III) were restored by incremental technique with 2-mm increments of nanofilled resin

composite (Flitek™Z350 XT Universal restorative, 3M ESPE) and light cured for 20s on each layer. For the group IV and V, the cavities were treated with 37 % phosphoric acid (Scotchbond™Etching liquid, 3M ESPE) and the bonding agent (Adper™ Single Bond^2, 3M ESPE) as mentioned. The group IV was bulkily restored with bulk fill resin composite (Filtek™ Bulk Fill Posterior Restorative, 3M ESPE). The group V was incrementally restored with the nanofilled resin composite (Flitek™Z350 XT Universal restorative, 3M ESPE).

After the tofflemire matrix holder and metal band were removed, all samples were light cured for 20s at buccal and palatal aspects by using LED light curing unit (Mini LED ACTEON, France) with light intensity 2,000 mW/cm^2. Then all samples were stored in distilled water at 37°C for 24h and subjected to thermal cycling for 2000 cycles with temperature range of 5°C to 55°C with dwell time of 15s and 7s transferred time.[30,31]

2. Nanoleakage evaluation

The samples in group I, II and III were coated with two layers of nail varnish excepted 1 mm surrounding the liner material and 1 mm around the cervical dentin margin in group IV and V. All samples were immersed in a 50 % ammoniacal silver nitrate solution (pH=9.5) for 24 h in the dark. Then, they were thoroughly rinsed with distilled water and immersed in a photo-developing solution for 8 h under fluorescent light to reduce diamine silver ions to metallic silver grains.[32]

The samples were fixed in 2.5 % glutaraldehyde in 0.1 M PBS buffer at pH 7.4 for 12 h at 4°C. After fixation, the specimens were rinsed by distilled water for 1 min. The samples were longitudinally sectioned in a mesio-distal direction through the center of the restorations. They were processed,[33] mounted on aluminum stubs, sputter-coated with gold and observed under SEM using backscattered electron mode (magnification x1000).

3. Data analysis

The distance of silver nitrate deposition along the gingival floor was measured from the SEM micrographs by Image J software program. The extension of nanoleakage was calculated as the percentage of silver nitrate deposition on the gingival floor. In addition, the SEM micrographs were measured the gap width in three points of each sample (inner point, middle point and outer point of silver nitrate deposition). Normal distribution was verified with the Shapiro-Wilk test and homogeneity by Levene's test. The mean percentages of silver nitrate deposition among groups were compared by Kruskal-Wallis Test. The mean gap widths at the gingival floor among groups were compared by Kruskal-Wallis Test followed by Mann Whitney U test. (p<0.05).

Results

The SEM micrographs presented the thickness of hybrid layer, the pattern of silver nitrate deposition and the gap formation (Fig. 1). In group I, II, IV and V the silver nitrate deposited entire thickness of hybrid layer, penetrated into the dentinal tubules and deposited on the resin tags. The inner and middle areas of the gingival wall presented the thicker hybrid layers compared with that of the outer area. In group III, all specimens presented modified hybrid layer which is clearly thinner than the hybrid layers of group I, II, IV and V. These modified hybrid layer had the silver nitrate deposition and it also penetrated into the dentinal tubules.

The percentages of silver nitrate deposition awere shown in Table 1. Group V (control) had the highest percentage of silver nitrate deposition (70.44 %), followed by group I (64.78 %), III (62.49 %), IV (58.96 %) and II (55.29 %). However there was no statistically significant difference among the restorative materials (p>0.05).

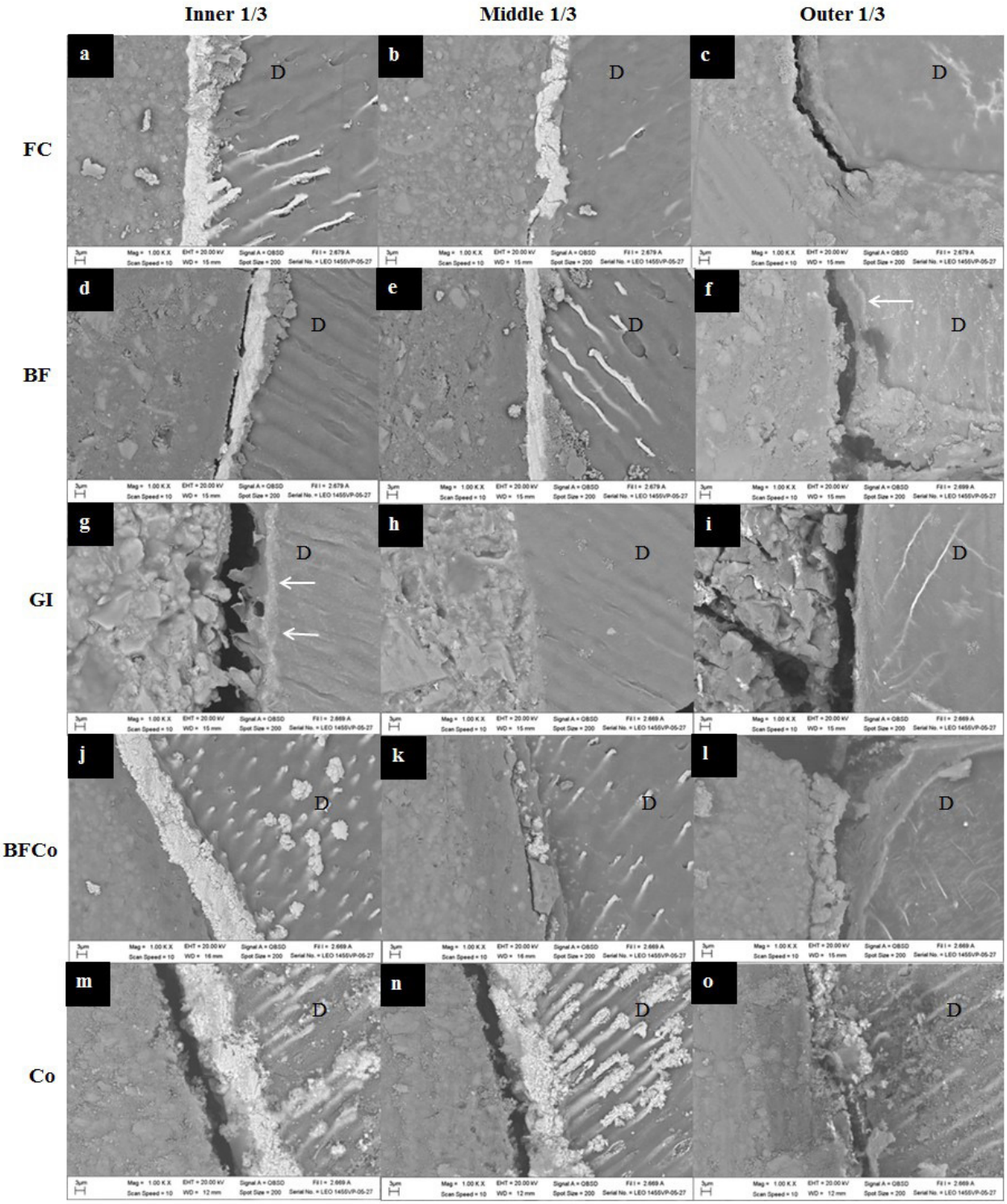

Figure 1 *The micrographs from SEM (1000x) present the thickness of hybrid layer, pattern of silver nitrate deposition and gap formation. Silver nitrate has a similar deposition pattern in all groups. It deposits in the dentinal tubules, resin tags and entire thickness of hybrid layer. Gaps are found between hybrid layer and bonding layer. Some areas have a silver nitrate deposition at the base of hybrid layer (arrow). The GI group shows the modified hybrid layer. The density of silver nitrate deposition in this group is clearly lower than others. (D=Dentin, FC=conventional flowable resin composite, BF=bulk fill flowable resin composite, GI=resin modified glass ionomer cement, BFCo = bulk fill resin composite, Co=conventional nanofilled resin composite)*

Table 1 *The mean and standard deviation (SD) of percentage of silver nitrate deposition at the cervical dentin*

Type of restoration	N	% of silver nitrate deposition Mean (SD)
Group I (FC+Co)	5	64.78 (14.14)[a]
Group II (BF+Co)	5	55.29 (13.47)[a]
Group III (GI+Co)	4	62.49 (4.14)[a]
Group IV (BFCo)	5	58.96 (1.46)[a]
Group V (Co)	5	70.44 (16.37)[a]

Lower case characters represent statistically significant differences ($p < 0.05$)

The gap formation was found in almost all outer area of samples. The gap formation presented between the hybrid layer or modified hybrid layer and materials (Fig. 1). The width of gap was different among materials and the positions in the cavity as shown in table 2.

Table 2 *The mean and standard deviation (SD) of the gap width at the cervical dentin margin*

Type of restoration	N	Gap width between hybrid layer & liner mean (SD), μm		
		Outer	Middle	Inner
Group I (FC+Co)	5	5.23 (2.94)[A,a]	0.41 (0.92)[B,a,b]	0.00 (0.00)[B,a]
Group II (BF+Co)	5	1.35 (1.30)[A,a]	0.00 (0.00)[A,a]	0.18 (0.26)[A,a]
Group III (GI+Co)	4	2.70 (3.12)[A,a]	0.00 (0.00)[A,a]	4.45 (2.36)[A,b]
Group IV (BFCo)	5	1.73 (0.91)[A,a]	0.00 (0.00)[B,a]	0.00 (0.00)[B,a]
Group V (Co)	5	3.88 (1.56)[A,a]	1.18 (0.74)[A,b]	2.38 (2.19)[A,a,b]

Lower case characters represent statistically significant differences ($p<0.05$) within columns

Upper case characters represent statistically significant differences ($p<0.05$) within rows

The group I, II, IV and V had the largest gaps in the outer area (5.23, 1.35, 1.73 and 3.88 μm respectively) while the inner area of group III showed the largest ones (4.45 μm). The gap width of the outer area of group I and IV was significantly larger than other areas (p=0.043). However only group IV did not present gap formation at inner and middle area. In the group III had significantly larger size of gap at the inner area when compared with group I, II and IV (p=0.016). There was no gap formation in the middle area in all samples in group II, III and IV. The group V had the significantly larger size of gap in the middle when compared with group II, III and IV (p=0.032).

The simple correlation analysis presented no correlation between the mean percentage of silver nitrate deposition and the gap width at gingival floor among five groups (p>0.05) (data not shown).

Discussion

The silver nitrate deposition represents the incomplete bonding of either an adhesive systems or restorative materials. These defects occur as the nanometer-sized spaces around the collagen fibrils within the hybrid layer. They are a result of incomplete infiltration of adhesive resin into a demineralized dentin.[22-24,34,35]

The percentages of silver nitrate deposition among three lining materials in class II resin composite open sandwich technique were no statistical difference. The pattern of silver nitrate deposition in group I, II, IV and V had the similar pattern because these four groups used the same adhesive system (Single Bond2). The degree of polymerization shrinkage of all resin-based materials did not influence on the nanoleakage of bonding agent.

The majority of silver nitrate deposited at the dentin side of hybrid layer. This probably denoted the accumulation of shrunk collagen fibers on the dentin surface after applying etchant.[34] This thin layer (0.2-0.3 μm) might interfere the adhesive resin infiltration, consequently the silver ion precipitation.[22] In addition, the components of the adhesive reagent probably affected the silver nitrate deposition. The adhesives with high percentage of hydrophilic monomers demonstrated a high degree of permeability after polymerization and the silver nitrate deposition.[36] The Single Bond2 containing HEMA, which is hydrophilic monomer, improves their infiltrating ability into the moist substrate. However, HEMA has low water vapor pressure, so water commonly retains in the layers which have the adhesive reagent. Consequently, the hybrid layer acted as a hydrogel which promotes silver nitrate deposition.[37]

Some specimens presented the silver nitrate deposition at the material side of hybrid layer. This result was similar to the previously studies.[24,38-40] Van Meerbeek *et al.* found an amorphous electron-dense phase on the top of the hybrid layer for Scotchbond Multi-Purpose, which contained polyalkenoic acid. They suggested that it represented a phase separation of the polyalkenoic acid copolymer from the other primer ingredients, which reacted with calcium to form calcium-polycarboxylate salts.[39,40] In addition, Vargas *et al.* found amorphous hybrid layer-like structures above the hybrid layer in SEM observations, for Single Bond and Scotchbond Multi-Purpose.[38] Li *et al.* observed the amorphous structure in Single Bond and One Coat Bond, which contain polyalkenoic acid. This amorphous structure uptake silver ions on the top of the hybrid layer.[24]

Almost all samples showed the thick hybrid layer and silver nitrate deposition at the inner area. The air blowing for evaporating solvent probably caused the accumulation of adhesive resin at the inner line angle. The thick layers of adhesive resin might prevent the proper evaporation of solvent, resulting in poor polymerization.[41] The residual monomer probably caused the infiltration of silver nitrate within the resin[42,43] consequently the deposition of silver nitrate within a hybrid layer or entire thickness of hybrid layer. Additionally, the moist bonding technique might leave the excess water along the line angle interfering the evaporation of solvent and resulting in the incomplete polymerization of resin.

The discontinuous silver nitrate deposition within hybrid layer may be indicated that the hybrid layer is not uniform. Some parts of hybrid layer are probably well polymerized and others poorly polymerized.

The group III (GI+Co) showed the deposition of silver nitrate within the modified hybrid layer and within the mass of the resin modified glass ionomer cement. The hydrophilic functional monomers contained in resin modified glass ionomer cement can absorb water resulting in hydrolytic degradation and silver nitrate deposition.[44] In addition, the porosity of material probably causes silver nitrate deposition within the material mass.[45,46]

Almost all specimens demonstrated a gap formation at the outer area of the cervical dentin. The

gap width of the outer area of group I (5.23 μm) was larger than that of group V (control) (3.88 μm). The gap width of the outer area of group II (1.35 μm), III (2.70 μm) and IV (1.73μm) were smaller than that of group V (control). However they were insignificantly different. These results might imply that the bulk fill flowable resin composite and the resin modified glass ionomer cement using as the liners in the class II resin composite open sandwich technique might be able to improve the adaptability of the restoration at the outer area of the internal wall. In the other hand, applying the conventional flowable resin composite as the liner in the class II resin composite open sandwich technique cannot improve the marginal adaptability. Moreover this present study advocated that the bulk fill resin composite (either flowable bulk fill or conventional bulk fill resin composites) with the bulk filled technique can improve the marginal adaptability.

Regarding the inner area, the gap width of group III was insignificantly larger than the control group. The RMGI material is more viscous than the bonding agent, which has the chemical bond to resin composite materials. The flow rate of RMGI is low, so its adaptability is less than the bonding agent. The inner area is the most critical area for the adaptability of filling materials. When the gap is formed at this area, the restorative material is more susceptible to the hydrolytic degradation. From this issue, it might need further studies for the degradation of RMGI due to the large gap formation at the inner area.

The conventional flowable resin composite has a low modulus of elasticity. A placement of flowable resin composite as a lining material can dissipate stress and reduce shrinkage stress of resin composite restorative material at tooth-restoration interface.[8,47,48] In addition, the flowable resin composite has low surface tension, therefore this material can penetrate into the irregularity surface resulting in better adaptability.[9-11,49] In the other hand, the conventional flowable resin composite contains 20-25 % less filler than conventional materials and larger amount of diluent monomers resulting in high polymerization shrinkage.[16,50] The diluent monomer especially TEGDMA, which contains in Filtek™ Z350 XT flowable, has a small molecule with more active sites leading to negative effect on polymerization shrinkage.[51]

The results from this study were similar to the previous ones which reported that the bulk fill flowable resin composites and high viscosity bulk fill resin composites showed better dentin marginal adaptation and less gap formation compared with conventional flowable resin composite.[19,21,30] The bulk fill resin composites demonstrated the low polymerization shrinkage stress and the high degree of light transmission because of the reduction of light scattering at filler-matrix interface by either reducing the filler contents or increasing the filler particle size.[17,18] The SureFil SDR Flow® was added a modified urethane dimethacrylate in an organic part together with the photoactive groups leading to the reduction of the shrinkage stress.[52] The occurrence of gap formation might be related with the polymerization shrinkage of materials. The SureFil SDR Flow® has small gap formation compared with others probably due to low polymerization shrinkage. Nevertheless, the bulk fill flowable resin composite has significantly lower mechanical properties compared with the high viscosity bulk fill nanohybrid and conventional flowable resin composite.[18,52] Therefore, the manufacturers recommend using this material as the intermediate layer.

The Filtek™ Bulk fill Posterior restorative is a high viscosity bulk fill resin composite. It contains two novel methacrylate monomers; a high molecular weight aromatic dimethacrylate (AUDMA) and an additional fragmentation monomer (AFM). The AUDMA has less reactive groups than conventional dimetahcrylate monomer. This might decrease the polymerization shrinkage of polymers. The AFM has a reactive site to cleave through a fragmentation process during polymerization. This can lead to the stress relief after polymerization.[53]

The resin modified glass ionomer cement was an alternative lining material for class II resin composite

open sandwich technique which may improve the marginal adaptability regarding to the outer area.[6,7,54,55] However the longevity of RMGI is likely to have further study due to the large gap formation at the inner area.

Discussion

Type of resin-base materials has no influence on the nano-leakage of restorative materials at the cervical dentin of class II cavity when using the same kinds of bonding agent. The bulk fill flowable resin composite (SureFil SDR Flow®) and the high viscosity bulk fill resin composite (Filtek™ Bulk fill Posterior restorative) can improve the marginal adaptability at the cervical dentin of class II cavity.

Acknowledgement

We are grateful for the student's research grant supported by the Graduate School of Naresuan University and greatly acknowledge 3M/ESPE, ACCORD Corporation Company Limited and Dentsply (Thailand) for kindness to support the restorative materials for this study.

Reference

1. Van Meerbeek B, De Munck J, Yoshida Y, Inoue S, Vargas M, Vijay P, *et al.* Buonocore memorial lecture. Adhesion to enamel and dentin: current status and future challenges. ***Oper Dent*** 2003;28:215-35.

2. Bausch JR, de Lange K, Davidson CL, Peters A, de Gee AJ. Clinical significance of polymerization shrinkage of composite resins. ***J Prosthet Dent*** 1982;48:59-67.

3. Dietschi D, De Siebenthal G, Neveu-Rosenstand L, Holz J. Influence of the restorative technique and new adhesives on the dentin marginal seal and adaptation of resin composite Class II restorations: an *in vitro* evaluation. ***Quintessence Int*** 1995;26:717-27.

4. Mahrous AI, Eltiti HA, Ahmed IM, Alagha EI. Effect of different gingival margin restorations of class II cavities on microleakage: an *in-vitro* study. ***Electron Physician*** 2015;7:1435-40.

5. Tredwin CJ, Stokes A, Moles DR. Influence of flowable liner and margin location on microleakage of conventional and packable class II resin composites. ***Oper Dent*** 2005; 30:32-8.

6. Aggarwal V, Singla M, Yadav S, Yadav H. Effect of flowable composite liner and glass ionomer liner on class II gingival marginal adaptation of direct composite restorations with different bonding strategies. ***J Dent*** 2014;42:619-25.

7. Dietrich T, Losche AC, Losche GM, Roulet JF. Marginal adaptation of direct composite and sandwich restorations in Class II cavities with cervical margins in dentine. ***J Dent*** 1999;27:119-28.

8. Karaman E, Ozgunaltay G. Polymerization shrinkage of different types of composite resins and microleakage with and without liner in class II cavities. ***Oper Dent*** 2014;39:325-31.

9. Sadeghi M, Lynch CD. The effect of flowable materials on the microleakage of Class II composite restorations that extend apical to the cemento-enamel junction. ***Oper Dent*** 2009;34:306-11.

10. Leevailoj C, Cochran MA, Matis BA, Moore BK, Platt JA. Microleakage of posterior packable resin composites with and without flowable liners. ***Oper Dent*** 2001;26:302-7.

11. Civelek A, Ersoy M, L'Hotelier E, Soyman M, Say EC. Polymerization shrinkage and microleakage in Class II cavities of various resin composites. ***Oper Dent*** 2003; 28:635-41.

12. Yoshikawa T, Sano H, Burrow MF, Tagami J, Pashley DH. Effects of dentin depth and cavity configuration on bond strength. ***J Dent Res*** 1999;78:898-905.

13. Tanumiharja M, Burrow MF, Cimmino A, Tyas MJ. The evaluation of four conditioners for glass ionomer cements using field-emission scanning electron microscopy. ***J Dent*** 2001;29:131-8.

14. Soubhagya M, Goud KM, Deepak BS, Thakur S, Nandini TN, Arun J. Comparative *in vitro* evaluation of internal adaptation of resin-modified glass ionomer, flowable

composite and bonding agent applied as a liner under composite restoration: A scanning electron microscope study. ***J Int Oral Health*** 2015;7:27-31.
15. Miguez PA, Pereira PN, Foxton RM, Walter R, Nunes MF, Swift EJ Jr. Effects of flowable resin on bond strength and gap formation in Class I restorations. ***Dent Mater*** 2004;20:839-45.
16. Pecie R, Onisor I, Krejci I, Bortolotto T. Marginal adaptation of direct class II composite restorations with different cavity liners. ***Oper Dent*** 2013;38:E210-20.
17. Kim RJ, Kim YJ, Choi NS, Lee IB. Polymerization shrinkage, modulus, and shrinkage stress related to tooth-restoration interfacial debonding in bulk-fill composites. ***J Dent*** 2015;43:430-9.
18. Bucuta S, Ilie N. Light transmittance and micro-mechanical properties of bulk fill vs. conventional resin based composites. ***Clin Oral Investig*** 2014;18:1991-2000.
19. Scotti N, Comba A, Gambino A, Paolino DS, Alovisi M, Pasqualini D, *et al.* Microleakage at enamel and dentin margins with a bulk fills flowable resin. ***Eur J Dent*** 2014;8:1-8.
20. Ilie N, Hickel R. Investigations on a methacrylate-based flowable composite based on the SDR technology. ***Dent Mater*** 2011;27:348-55.
21. Agarwal RS, Hiremath H, Agarwal J, Garg A. Evaluation of cervical marginal and internal adaptation using newer bulk fill composites: An *in vitro* study. ***J Conserv Dent*** 2015;18:56-61.
22. Sano H, Takatsu T, Ciucchi B, Horner JA, Matthews WG, Pashley DH. Nanoleakage: leakage within the hybrid layer. ***Oper Dent*** 1995;20:18-25.
23. Sano H, Shono T, Takatsu T, Hosoda H. Microporous dentin zone beneath resin-impregnated layer. ***Oper Dent*** 1994;19:59-64.
24. Li H, Burrow MF, Tyas MJ. Nanoleakage patterns of four dentin bonding systems. ***Dent Mater*** 2000;16:48-56.
25. de Mattos Pimenta Vidal C, Pavan S, Briso AL, Bedran-Russo AK. Effects of three restorative techniques in the bond strength and nanoleakage at gingival wall of Class II restorations subjected to simulated aging. ***Clin Oral Investig*** 2013;17:627-33.
26. Kohn WG, Harte JA, Malvitz DM, Collins AS, Cleveland JL, Eklund KJ. Guidelines for infection control in dental health care settings--2003. ***J Am Dent Assoc*** 2004;135:33-47.
27. Lee JJ, Nettey-Marbell A, Cook A Jr, Pimenta LA, Leonard R, Ritter AV. Using extracted teeth for research: the effect of storage medium and sterilization on dentin bond strengths. ***J Am Dent Assoc*** 2007;138:1599-603.
28. Attam K, Talwar S, Yadav S, Miglani S. Comparative analysis of the effect of autoclaving and 10 % formalin storage on extracted teeth: A microleakage evaluation. ***J Conserv Dent*** 2009;12:26-30.
29. DeWald JP. The use of extracted teeth for *in vitro* bonding studies: a review of infection control considerations. ***Dent Mater*** 1997;13:74-81.
30. Roggendorf MJ, Kramer N, Appelt A, Naumann M, Frankenberger R. Marginal quality of flowable 4-mm base vs. conventionally layered resin composite. ***J Dent*** 2011;39:643-7.
31. Wattanawongpitak N, Yoshikawa T, Burrow MF, Tagami J. The effect of thermal stress on bonding durability of resin composite adaptation to the cavity wall. ***Dent Mater J*** 2007;26:445-50.
32. Peliz MI, Duarte S Jr, Dinelli W. Scanning electron microscope analysis of internal adaptation of materials used for pulp protection under composite resin restorations. ***J Esthet Restor Dent*** 2005;17:118-28.
33. Tay FR, Pashley DH, Yoshiyama M. Two modes of nanoleakage expression in single-step adhesives. ***J Dent Res*** 2002;81:472-6.
34. Pashley DH, Ciucchi B, Sano H, Horner JA. Permeability of dentin to adhesive agents. ***Quintessence Int*** 1993; 24:618-31.
35. Eick JD, Gwinnett AJ, Pashley DH, Robinson SJ. Current concepts on adhesion to dentin. ***Crit Rev Oral Biol Med*** 1997;8:306-35.
36. Tay FR, Pashley DH, Suh BI, Carvalho RM, Itthagarun A. Single-step adhesives are permeable membranes. ***J Dent*** 2002;30:371-82.
37. Tay FR, King NM, Chan KM, Pashley DH. How can

nanoleakae occur in self-etching adhesive systems that demineralize and infiltrate simultaneously? ***J Adhes Dent*** 2002;4:255-69.
38. Vargas MA, Cobb DS, Denehy GE. Interfacial micromorphology and shear bond strength of single-bottle primer/adhesives. ***Dent Mater*** 1997;13:316-24.
39. Van Meerbeek B, Conn LJ Jr, Duke ES, Eick JD, Robinson SJ, Guerrero D. Correlative transmission electron microscopy examination of nondemineralized and demineralized resin-dentin interfaces formed by two dentin adhesive systems. ***J Dent Res*** 1996;75:879-88.
40. Van Meerbeek B, Yoshida Y, Lambrechts P, Vanherle G, Duke ES, Eick JD, *et al.* A TEM study of two water-based adhesive systems bonded to dry and wet dentin. ***J Dent Res*** 1998;77:50-9.
41. Zheng L, Pereira PN, Nakajima M, Sano H, Tagami J. Relationship between adhesive thickness and microtensile bond strength. ***Oper Dent*** 2001;26:97-104.
42. Ferracane JL. Elution of leachable components from composites. ***J Oral Rehabil*** 1994;21:441-52.
43. Lee SY, Greener EH, Menis DL. Detection of leached moieties from dental composites in fluids simulating food and saliva. ***Dent Mater*** 1995;11:348-53.
44. Cattani-Lorente MA, Godin C, Meyer JM. Mechanical behavior of glass ionomer cements affected by long-term storage in water. ***Dent Mater*** 1994;10:37-44.
45. Alster D, Feilzer AJ, De Gee AJ, Mol A, Davidson CL. The dependence of shrinkage stress reduction on porosity concentration in thin resin layers. ***J Dent Res*** 1992;71:1619-22.
46. Miyazaki M, Fukuishi K, Onose H. Influence of light irradiation on the volumetric change of polyacid-modified resin composites. ***J Dent*** 1999;27:149-53.
47. Olmez A, Oztas N, Bodur H. The effect of flowable resin composite on microleakage and internal voids in class II composite restorations. ***Oper Dent*** 2004;29:713-9.
48. Basavanna R, Garg A, Kapur R. Evaluation of gingival microleakage of class II resin composite restorations with fiber inserts: An *in vitro* study. ***J Conserv Dent*** 2012;15:166-9.
49. Estafan D, Estafan A, Leinfelder KF. Cavity wall adaptation of resin-based composites lined with flowable composites. ***Am J Dent*** 2000;13:192-4.
50. Xavier JC, Monteiro GQdM, Montes MAJR. Polymerization Shrinkage and Flexural Modulus of Flowable Dental Composites. ***Mater Res*** 2010;13:380-4.
51. Asmussen E, Peutzfeldt A. Influence of UEDMA BisGMA and TEGDMA on selected mechanical properties of experimental resin composites. ***Dent Mater*** 1998;14:51-6.
52. Ilie N, Bucuta S, Draenert M. Bulk-fill resin-based composites: an *in vitro* assessment of their mechanical performance. ***Oper Dent*** 2013;38:618-25.
53. Cramer NB, Stansbury JW, Bowman CN. Recent advances and developments in composite dental restorative materials. ***J Dent Res*** 2011;90:402-16.
54. Sampaio PC, de Almeida Junior AA, Francisconi LF, Casas-Apayco LC, Pereira JC, Wang L, *et al.* Effect of conventional and resin-modified glass-ionomer liner on dentin adhesive interface of Class I cavity walls after thermocycling. ***Oper Dent*** 2011;36:403-12.
55. Goldman M. Polymerization shrinkage of resin-based restorative materials. ***Aust Dent J*** 1983;28:156-61.

Original Article

The Inhibition of Dental Caries Pathogen by Using Prebiotic and Probiotic Combination

Santichai Nunpan[1], Chatrudee Suwannachart[2] and Kornchanok Wayakanon[1]

[1]Department of Restorative Dentistry, Faculty of Dentistry, Naresuan University, Phitsanulok

[2]Biodiversity Research Centre, Thailand Institute of Scientific and Technological Research, Pathum Thani

Abstract

Dental caries is the most important global problem of oral disease. The local demineralization of tooth surface from an acid action is an initial step of the disease. The acids are produced when sugar from food has an interaction with bacteria in the dental plaque, which usually accumulates on the susceptible tooth surfaces. As more acidic condition, the aciduric and acidogenic bacteria can survive. *Streptococcus mutans* is a major contributor of tooth decay. Many strategies are recommended to protect the susceptible teeth from cariogenic bacteria. A probiotic application is one of techniques providing the health-beneficial microorganism to inhibit the cariogenic bacteria. Prebiotics are oligosaccharides which can promote the growth of probiotics in human bowel. The objective of this study was to evaluate the efficiency of the prebiotic (Galacto-oligosaccharides, (GOS)) to enhance the probiotic (*Lactobacillus acidophilus*) for inhibition of *S. mutans* and *L. acidophilus* were co-cultured with ratio of 1:20 in the de Man, Rogosa and Sharpe (MRS) media supplemented with different concentrations of GOS; 1, 2, 3 and 4 % (v/v). The efficiency of synbiotic against *S. mutans* was determined from their growth rate. The growth rate of *S. mutans* and *L. acidophilus* were similar (0.4848 and 0.4861 hr^{-1}, respectively) in the MRS agar without GOS. The growth rate of *S. mutans* insignificantly decreased when grew in 3 and 4 % of GOS (0.1719 and 0.3258 hr^{-1} respectively) compared with control group ($p > 0.05$), while the growth rate of *L. acidophilus* was constant (0.3443 and 0.3459 hr^{-1} respectively). The GOS was not an efficient prebiotic to enhance the function of *L. acidophilus* to inhibit growth of *S. mutans*.

Keywords: Probiotic, Prebiotic, Galacto-oligosaccharide, *L. acidophilus*, *S. mutans*, Dental caries

Received Date: APR 27, 2017 **Accepted Date:** JUL 17, 2017

doi: 10.14456/jdat.2017.19

Correspondence to:

Kornchanok Wayakanon, Department of Restorative Dentistry, Naresuan University, Phitsanulok, 65000 Thailand Tel. 094-336-4994

E-mail: wkornchanok@hotmail.com

Introduction

Dental caries is a major oral infectious disease initiated with the local demineralization by acids which are produced from accumulated acidogenic bacteria in the dental plaque.[1] The dental plaque presents as a diverse community of microorganisms and extracellular matrix in an arranged biofilm. The microorganisms tightly attach each other to form an open architecture with channels and voids for the circulation of nutrients, waste products and gas.[2]

Acids are produced from the consumption of sugar by bacteria. It is a cause of the dissolution of the mineral composition of tooth leading to dental decay. The key pathogens in this mechanism are acidogenic bacteria such as *S. mutans*, *Actinomyces* spp. and *Lactobacillus* spp.

Streptococcus mutans is facultative anaerobic Gram-positive cocci. In human saliva, the number of them normally ranges from undetectable to 10^6 -10^7 CFU/ml. They can consume several kinds of sugar resulting in the production of several weak acids particularly lactic acid together with the linear-soluble and branched-insoluble exopolysaccharides (EPS), such as glucans and fructans, by glucosyltransferase (GTFs) and fructosyltransferase (FTFs) for cell adhesion.[2,3] These exopolysaccharides are used to facilitate the adhesion of the second colonizers, the stability of biofilm and the protection of bacteria from host defense mechanism.[1,2,3] The restriction of the number of *S. mutans* is one of plenty strategies for the caries prevention.[6,7]

Probiotics are living microorganisms providing health benefits to the hosts. They are naturally found in a human body and have an influence on other microorganisms by producing the specific antimicrobial substances.[5] In the oral cavity, the probiotics must first attach to oral tissue followed by creating a protective barrier to prevent the pathogenic microorganism colonization. They must increase in number in order to produce the effective capacity.[5,8] The most well-known probiotics are *Lactobacillus* spp. and *Bifidobacterium* spp. The consumption of probiotics approximately 10^7 – 10^9 CFU/ml of *Lactobacillus* spp. is adequate to modulate the benefit to intestine microflora.[9-11]

Lactobacillus spp. is Gram-positive, non-spore forming bacilli which normally isolated from gastrointestinal tract of human.[4,5] The optimal growth condition is between 30 – 40 °C, 5 % CO_2 and in the growth media pH 5.5–5.8. *Lactobacillus acidophilus* produce several kinds of bacteriocins; such as Lactacin B, Lactacin F, Brevicin 37 and Gassericin A, which affect specific strains in complex microbial biofilm.[4] *Lactobacillus casei* strain GG could produce various antimicrobial components such as organic acids, hydrogen peroxide, carbon peroxide, diacetyl, low molecular weight antimicrobial substances, bacteriocins, and adhesion inhibitors against *Streptococcus* spp.[12,13] According to Kojima Y. *et al.* (2015), *Lactobacillus* spp. can also inhibit the insoluble glucan formation of *S. mutans*.[14] Some *Lactobacillus* spp. have been considered as potential probiotics.[15]

Prebiotics have been defined as non-digestible food ingredients which beneficially affect the host by selectively stimulating the growth or activity of microorganisms in the human colon. The most common prebiotics are non-digestible polysaccharides such as lactosucrose, fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS) and isomalto-oligosaccharides.[16] However, only FOS, GOS and inulin have been tested *in vivo* to meet all the requirements for current criteria of prebiotics.[17]

The probiotics have the negative effects on the cariogenic bacteria whilst the prebiotics selectively promote the growth or activities of the probiotics. The efficient combination between them is named synbiotics.[20] The synbiotics, an enhanced probiotics by prebiotics, provide beneficially effects on the hosts.[21] Culture *L. acidophilus* with conjac glucomanan as prebiotics was able to inhibit the growth of *S. mutans*.[22] According to Kondepudi *et al.* (2012), GOS increased growth rates of

Bifidobacterium breve, Bifidobacterium longum and *Bifidobacterium pseudocatenulatum*.[23] However, there are few studies of probiotics and prebiotics in dental caries modulation.[24] The aim of this study was to investigate the inhibitory effect of synbiotics between GOS and *L. acidophilus* (TISTR 2365T = DSMZ 20079T) on the growth of *Streptococcus mutans* (DSMZ 20523T).

Materials and Methods

1. Preparation of culture medium, prebiotic and microorganism

1.1 Culture medium preparation

Three culture media (Brain heart infusion broth (BHI), de Man Rogosa and Sharpe broth (MRS) and tryptic soy broth (TSB) were used for culture optimization. The composition of each culture media was prepared following the manufacturer's recommendation. For agar medium preparation, 1.5 % of agar powder was added into the culture broth. All prepared media were sterilized in an autoclave at 121°C, pressure 15 lb/inch^2 for 15 minutes.

1.2 Prebiotic preparation

The galacto-oligosaccharides (GOS) (Bornnet corporation Co., Ltd., Bangkok, Thailand) was prepared at concentrations 1 %, 2 %, 3 % and 4 % (v/v) in MRS broth.

1.3 Microorganism preparation

The microorganisms in this experiment were classified as cariogenic bacteria and probiotic. The cariogenic bacteria was *S. mutans* (DSMZ 20523T). The probiotic was *L. acidophilus* (TISTR 2365T or DSMZ 20079T). From the lyophilized stock, *S. mutans* and *L. acidophilus* were inoculated in the BHI and MRS broths respectively at 37°C, 5 % CO_2 for 18-24 hrs. The overnight cultures of each kind of bacteria were then inoculated on the BHI and MRS agars by the streak plate technique. An isolated colony was transferred to fresh media (BHI, MRS and TYE) and allowed to grow. Their growth patterns were recorded.

2. Determination of the efficacy of synbiotic on cariogenic bacteria

2.1 Determination of the suitable culture medium for co-culture

Five hundred microlitre of bacteria solution from section 1.3 was separately inoculated into 20 ml of three culture broths (BHI, MRS and TYE). The growth patterns of *S. mutans* and *L. acidophilus* in three culture broths were determined by the optical density (OD) at 600 nm along with serial dilution method for colony counting at 0, 1, 3, 6, 8, 10, 12, 14, 16, 18, 24, 36 and 48 hrs.

2.2 Determination of the proportion between *S. mutans* and *L. acidophilus* for co-culture.

S. mutans and *L. acidophilus* in the mid-log phase were used in the experiment. To determine the appropriate proportion for the equal number of *S. mutans* and *L. acidophilus*, they were co-cultured under various ratios; 1:5, 1:10, 1:20 and 1:40 in the MRS culture medium. Their growth patterns were investigated following section 2.1 in the MRS agar.

2.3 Effect of prebiotics on probiotics to inhibit *S. mutans*

The optimized proportion between *S. mutans* and *L. acidophilus* from the section 2.2 were co-cultured in MRS broth supplemented with different concentrations of GOS; 1, 2, 3 and 4 % (v/v). Their growth rates were calculated from the growth patterns, which were determined with the same fashion as section 2.1 in the MRS agar.

The bacteria growth rate was calculated from:

$\mu = ((\log 10\ N_t - \log 10\ N_0) \times 2.303)/ (t - t_0)$.[25]

μ : growth rate, N_t: the number of bacteria at t-log phase,

N_0: the number of bacteria at time point 0,

t : time point reached the mid-log phase

3. Data analysis

The experiments were performed triplicate to investigate the concentration of GOS that had the highest efficacy to enhance the growth rate or activity of *L.*

acidophilus to inhibit the growth of *S. mutans.* The growth rates of *S. mutans* and *L. acidophilus* from the co-culture with GOS-supplemented MRS media were compared by Kruskal-Wallis Test followed by Mann Whitney U test ($p < 0.05$) with alpha correction.

Results

1. Determination of the suitable culture medium for co-culture

S. mutans and *L. acidophilus* were grown in BHI, MRS and TYE broth to find the most appropriate one for both of them. *S. mutans* grew well in the MRS medium, but they stopped growing in BHI after 14 hrs and in TYE after 10 hrs. *L. acidophilus* required more nutrients to maintain their viability. They grew efficiently only in the MRS medium (Fig. 1). Thus, MRS medium was selected for the next experiments.

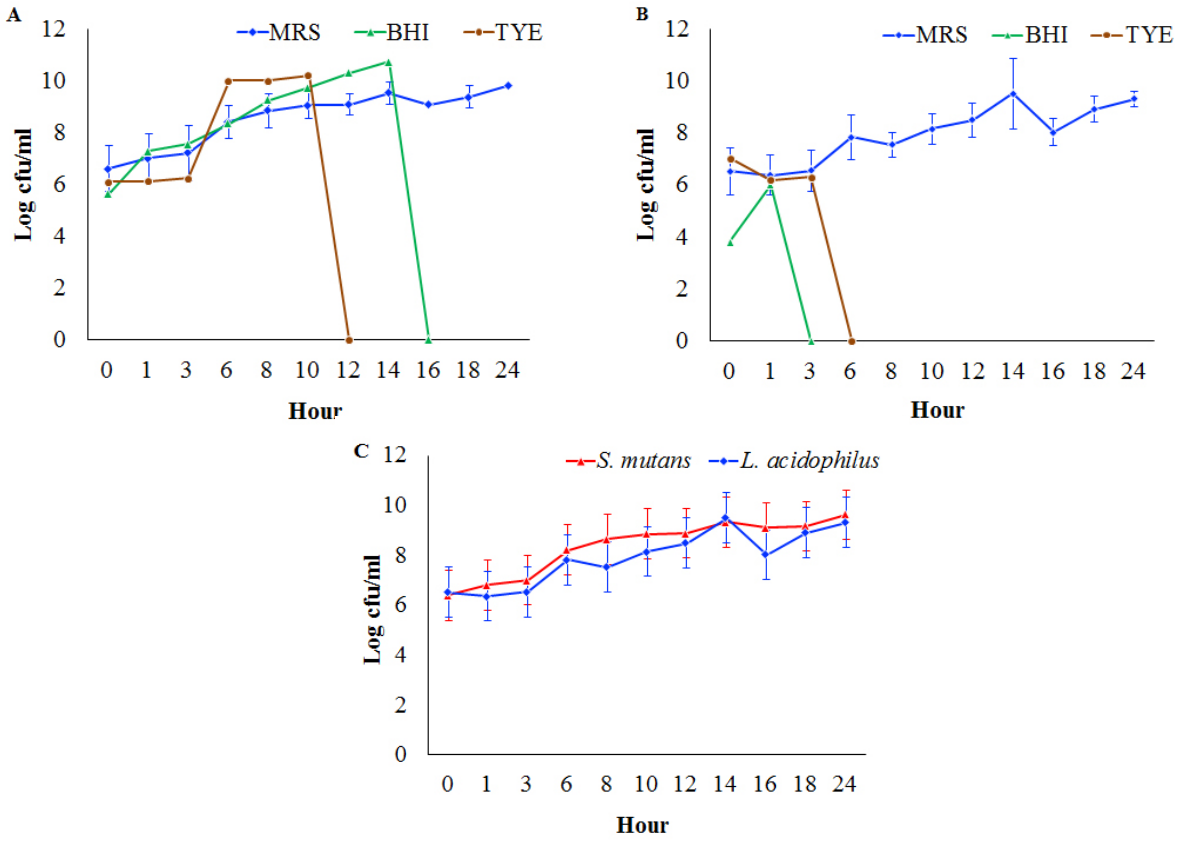

Figure 1 *The growth curves of S. mutans and L. acidophilus in MRS, BHI and TYE media are shown in 1a and 1b respectively. The individually growing capability of S. mutans and L. acidophilus in MRS broth is shown in 1C.*

2. Optimization of the inoculum ratio

From the section 1, the MRS medium was only one medium which both *S. mutans* and *L. acidophilus* could grow. When co-culture in the MRS broth and agar with the ratio 1:1, the number of living *S. mutans* was larger than that of *L. acidophilus* at the initial time point (data not shown). Therefore, the optimization for an equal number of both cells at the initial time point was performed.

S. mutans and *L. acidophilus* in the mid-log phase (OD 600 nm = 0.6) were selected. The various volume ratios between *S. mutans* and *L. acidophilus*;

1:5, 1:10, 1:20 and 1:40 were performed. The number of living *S. mutans* and *L. acidophilus* from the co-culture was similar (10^7 cells) in a ratio 1:20 and 1:40 at the initial time point. They had a similar growth pattern until 8 hrs. After that, *L. acidophilus* had the higher growth rate until 24 hrs (Fig. 2).

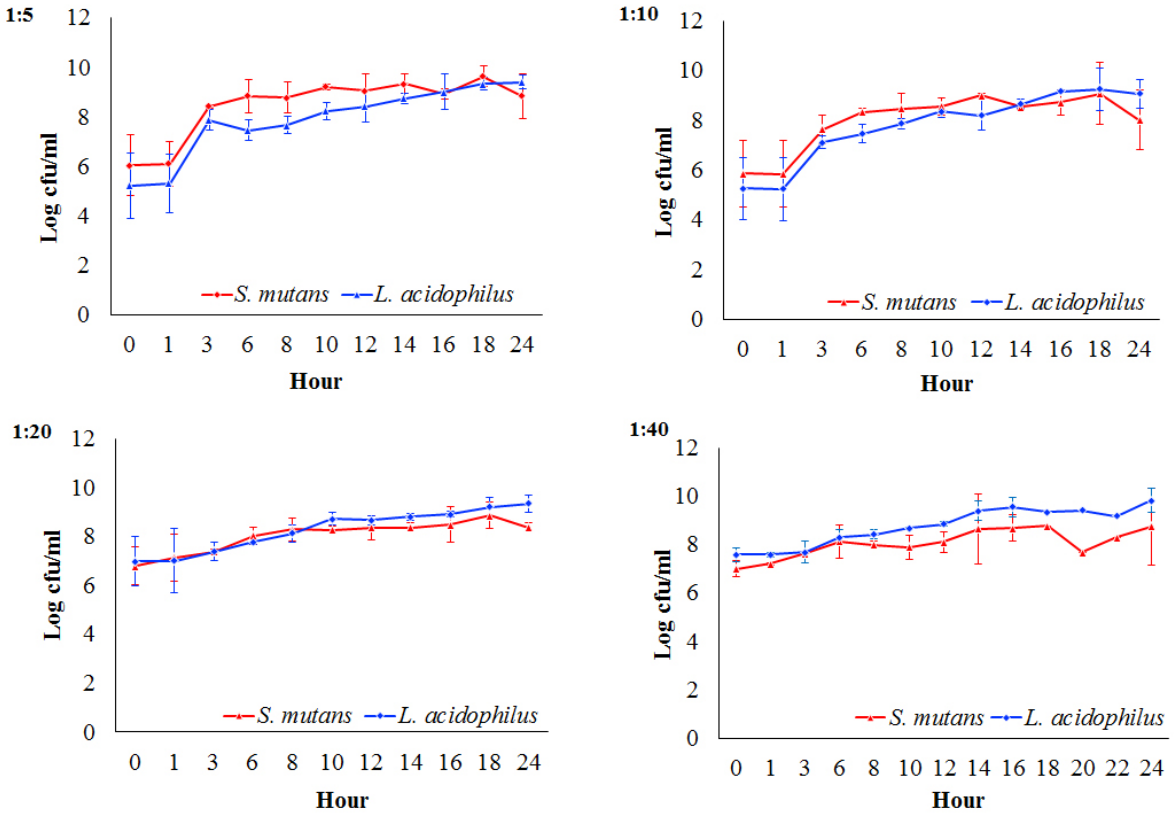

Figure 2 *The growth of S. mutans and L. acidophilus in different proportion*

S. mutans and *L. acidophilus* in the mid-log phase with the volume ratio 1:20 were selected for the next experiment since they had the equivalent number of living cells at the initial time point and their number of cells were more closely when compared with the ratio 1:40.

3. Determination of the inhibition effect of synbiotics towards *S. mutans*

S. mutans and *L. acidophilus* were cultured in the MRS broth supplemented with 1, 2, 3 and 4 % (v/v) of GOS. The growth patterns of them were determined by colony counting in the MRS agar. The maximum growth rate (at 6 hrs) was calculated from the growth patterns as in Fig. 3. Without GOS (control group), the growth rate of *S. mutans* and *L. acidophilus* were similar at 0.4848 and 0.4861 hr^{-1}, respectively.

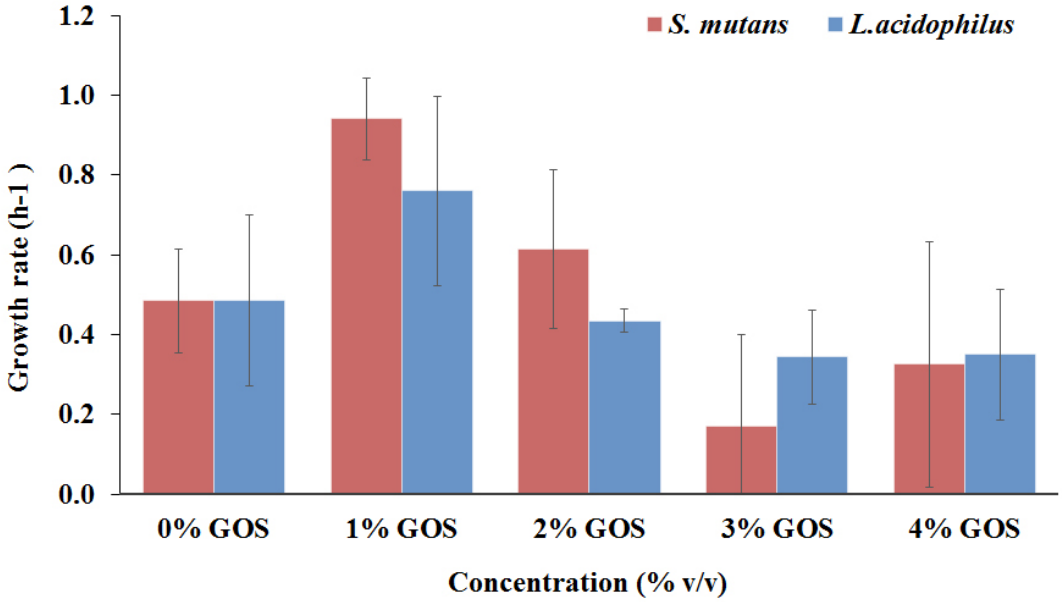

Figure 3 *The growth rates of S. mutans and L. acidophilus in different concentrations of GOS*

The growth rate of *S. mutans* increased in the culture presented 1 and 2 % of GOS (0.941 and 0.62 hr^{-1}). In 3 and 4 % of GOS, the growth rate of *S. mutans* decreased (0.1719 and 0.3258 hr^{-1}respectively) comparing to the control group (0.492 hr^{-1}) (*P*=0.057 and 0.857 respectively).

The growth rate of *L. acidophilus* slightly increased in the culture with 1 % of GOS (0.4861 hr^{-1}). When the concentration of GOS increased (2 %), the growth rates of *L. acidophilus* slightly decreased (0.4347 hr^{-1}) comparing to the control group and 1 % GOS group, but they were relatively constant at 3 and 4 % of GOS (0.3443 and 0.3499 hr^{-1} respectively).

Discussion

Prebiotics are oligosaccharides used to promote the functions of natural probiotics in human bowel. As in the oral cavity, dental caries is the major oral disease around the world. It has plenty of commensal microorganisms which are susceptible to function as pathogens with the alteration of ecology, similar to the human bowel. *L. acidophilus* has been known as probiotics in dental caries prevention. However there is no study to observe the efficiency of prebiotics in dental caries. This study investigated the effect of prebiotic (GOS) on *L. acidophilus* to inhibit growth of *S. mutans.* The suitable condition for co-culture needed to be determined. *L. acidophilus* normally prefer to grow in MRS broth while they can survive in BHI and TYE in just a short period of time. *L. acidophilus* require rich-amino acid and vitamin media. The MRS medium contains higher nutrients; such as peptone, beef extract, yeast extract including several salts (magnesium sulphate, manganese sulphate and dipotassium phosphate) to stimulate growth.[24] *S. mutans* can grow in either BHI or TYE and MRS broth while the maximum growth rate was observed in BHI medium at the first 14 hrs. Therefore MRS medium was selected for co-culture, *S. mutans* and *L. acidophilus.*

According to the growth curve of *S. mutans* and *L. acidophilus*, their logarithm phase were ranged from 6-8 hrs. At the mid-logarithm phase, the average number of *S. mutans* has shown about $2.7x10^8$ CFU/ml which was greater than *L. acidophilus* ($8.2x10^7$ CFU/ml). To determine the similar initial number of them, *S.*

mutans and *L. acidophilus* were co-culture in various ratios of 1:5, 1:10, 1:20 and 1:40. The number of *L. acidophilus* continuously increased to be larger than that of *S. mutans*. This result was corresponded with the previous studies of Singh *et al.* (2011)[26] and Nikawa *et al.* (2004).[27]

At 3 and 4 % of GOS in the co-culture, the number of *S. mutans* insignificantly decreased while there was fairly constant of that of *L. acidophilus.* The three percentages of GOS culture media was interesting for further studies in the synbiotic effect on *L. acidophilus.* Many studies found that *L. acidophilus* had ability to produce variety of antimicrobial substances such as Lactacin F and Lactacin B to compete growth of *S. mutans*.[24,28] The GOS might be able to activate the function (not the number) of *L. acidophilus* to inhibit the growth of *S. mutans.*

After 6 hrs. of the incubation, the number of *S. mutans* was gradually decrease while that of *L. acidophilus* was constant along with the pH of media drop to 5.3. *S. mutans* are the vital cariogenic initiators rather than *L. acidophilus*. When the number of *S. mutans* has been decreased, the dental caries might be difficult to occur. The pH of the co-culture medium dropped to 5.3 in this *in vitro* study. In the oral cavity, saliva has a buffering capacity to control the pH level. The pH level in the clinical situation is not likely to decline to the same level as the *in vitro* study. However the further studies are required.

GOS is a derivative of milk. It is commercially available as the ingredient in food for both infants and adults. It is not toxic when utilized for the clinical application. [29]

This is the preliminary study to investigate the synbiotic effect on the cariogenic bacteria. The prebiotic in this study showed some effects on the probiotic to against *S. mutans* even it was insignificant. The further studies were needed.

Conclusion

The GOS has no efficiency to enhance the function of *L. acidophilus* to inhibit the growth of *S. mutans* in this preliminary study.

Conclusion

This study was granted the FY2015 Thesis Grant for Master Degree Student from The National Research Council of Thailand. Many thanks to Dr. Pornsuda Nhawchai for statistic consultation.

References

1. Fejerskov O, Edwina K. Dental caries The Disease and its Clinical Management, ed. 2nd. 2008, Fusionopolis Walk: Blackwell Munksgaard Ltd. p. 4-5.

2. Marsh PD. Dental Plaque as a Microbial Biofilm. ***Caries Res*** 2004;38:204-211.

3. Richard JL, Jenkinson HF. Microbilogy of caries, in Oral Microbilogy at Glance. 2010, Blackwell Publishing: Fusionopolis Walk. p. 34-35.

4. Robinson RK, Batt CA. Encyclopedia of food microbiology, in Lactobacillus. 2000, Warszawa: Elsevier. p. 1134 - 1157.

5. Nair BG. and T.Y. Probiotic Foods in Health and Disease, 2011, New York: Science Publisher. p. 87 – 109.

6. Jingarwar MM, Bajwa NK, Pathak A. Minimal Intervention Dentistry- A review Frontier in Clinical Dentistry. ***J Clin Diagn Res*** 2014;8:ZE04-ZE08.

7. World Health Organization Food and Agriculture Organization of the United Nations. Probiotics in food: health and nutrition properties and guidelines for evaluation. FAO Food and Nutrition Paper. Rome, 2006.

8. Teughels W, Van Essche M, Sliepen I, Quirynen M. Probitics and Oral healthcare. ***Periodontol 2000*** 2008;48:111-47

9. Charteris WP, Kelly PM, Morelli L, Collins JK. Development and application of an *in vitro* methodology to determine the transit tolerance of potentially probiotic Lactobacillus and Bifidobacterium species in the upper human gastrointestinal tract. ***J Appl Microbiol*** 1998;84:759-68.
10. Fernández MF, Boris S, Barbés C. Probiotic properties of human lactobacilli strains to be used in the gastrointestinal tract. ***J Appl Microbiol*** 2003;94:449-55.
11. Pitino I, Randazzo CL, Cross KL, Parker ML, Bisignano C, Wickham MS, et al. Survival of Lactobacillus rhamnosus strains inoculated in cheese matrix during simulated human digestion. ***Food Microbiol*** 2012;31:57-63.
12. Silva M, Jacobus NV, Deneke C, Gorbach SL. Antimicrobial substance from a human Lactobacillus strain. ***Antimicrob Agents Chemother*** 1987;31:1231-3.
13. Hasslöf P, Hedberg M, Twetman S, Stecksén-Blicks C. Growth inhibition of oral mutans streptococci and candida by commercial probiotic lactobacilli - an *in vitro* study. ***BMC Oral Health*** 2010;10:18.
14. Kojima Y, Ohshima T, Seneviratne CJ, Maeda N. Combining prebiotic and probiotics to develop novel synbiotic that supress oral pathogen. ***Journal of oral bioscience*** 2015;58:27-32.
15. Aly SM, Abdel-Galil Ahmed Y, Abdel-Aziz Ghareeb A, Mohamed MF. Studies on Bacillus suntilis and Lactobacillus acidophilus, as potential probiotics, on the immune response and resistance of Tilapia nilotica (Oreochromis niloticus) to challange infection. ***Fish Shellfish Immunol*** 2008;25:128-36.
16. Sabater-Molina M, Larqué E, Torrella F, Zamora S. Dietary fructooligosaccharides and potential benefits on health. ***J Physiol Biochem*** 2009;65:315-28.
17. Roberfroid M. Prebiotics: The concept revisited. ***J Nutr*** 2007;137:830S-7S.
18. Brittany M. VBGCFJ. Prebiotics of Plant and Microbial Origin: Springer New York; 2011.)
19. Li W, Wang K, Sun Y, Ye H, Hu B, Zeng X. Influences of structures of galactooligosacharides and fructooligosacharide on the fermentation *in vitro* by human intestinal microbiota. ***J Funct Foods*** 2015;13:158-68.
20. Gibson GR, Probert HM, Loo JV, Rastall RA, Roberfroid MB. Dietary modulation of the human colonic microbiota: Updating the concept of prebiotics. ***Nutr Res Rev*** 2004;17:259-75.
21. Yukako K, Tomoko O, Chaminda J, Nobuko M. Combining prebiotics and probiotics to develop novel synbiotics that suppress oral pathogens. ***J Oral Biosci*** 2016;58:27-32.
22. Richard F, Farage H. A preliminary study of the synbiotic effects of conjac glucomannan hydrolysates (GMH) and lactobacilli on the the growth of the oral bacterium Streptococcus mutans. ***Nutrition & Food Science*** 2011;41.
23. Kondepudi KK, Ambalam P, Nilsson I, Wadström T, Ljungh A. Prebiotic-non-digestible oligosaccharides preference of probiotic bifidobacteria and antimicrobial activity against Clostridium difficile. ***Anaerobe*** 2012;18: 489-97.
24. Richard R, Batt CA, Pradip DP. Encyclopedia of food microbiology, in *Lactobacillus* 2000. The Bath Press: Great Britain. p. 1134 - 1157.
25. Neidhardt FC, Ingraham J, Schaechter M. Physiology of the bacterial cell, *a molecular approach*. 1990, Sunderland, Mass: Sinauer Associates. p. 485-490.
26. Singh RP, Damle SG, Chawla A. Salivary mutans streptococci and lactobacilli modulations in young children on consumption of probiotic ice-cream containing Bifidobacterium lactis Bb12 and Lactobacillus acidophilus La5. ***Acta Odontol Scand*** 2011;69:389-94
27. Nikawa H, Makihira S, Fukushima H, Nishimura H, Ozaki Y, Ishida K, *et al.* Lactobacillus reuteri in bovine milk fermented decreases the oral carriage of mutans streptococci. ***Int J Food Microbiol*** 2004;95:219-23.
28. Miriam BB, Julio PD, Sergio MQ, Carolina GL, Angel G. Probiotic mechanisms of action. ***Ann Nutr Metab*** 2012;61:160-174.
29. Gibson GR. Dietary modulation of the human gut microflora using prebiotics. ***Br J Nutr*** 1998;80:S209–12.

Original Article

Preliminary Study of LINE-1 Methylation Level in Long-term Cultivation of Human Dental Pulp Stem cells

Atitaya Vongprommool[1], Keskanya Subbalekha[1], Apiwat Mutirangura[2] and Prasit Pavasant[3]

[1]Department of Surgery, Faculty of Dentistry, Chulalongkorn University, Bangkok

[2]Department of Anatomy, Faculty of Medicine, Chulalongkorn University, Bangkok

[3]Department of Anatomy, Faculty of Dentistry, Chulalongkorn University, Bangkok

Abstract

Human dental pulp stem cells become an alternative source of stem cells that play an important role in tissue engineering and cell-based therapies that will help the regeneration of impaired tissue in elderly, regarding to the easy access and the potential to renew and differentiate into many cell types. Cell expansion *in vitro* is necessary to amplify the small amount of cells that can be isolated before therapeutic use of DPSC. However, long-term cultivation leads to the alteration in morphology, proliferation and differentiation potential of DPSCs. Moreover, the accumulation of mutation during cell expansion can bring the risk of malignant transformation. Epigenetic mechanism including DNA methylation was found taking part in the regulatory processes in development. The global loss of methylation level relates with many events including tumor progression. Hypomethylation of LINE-1 is associated with many types of cancer and occurs during early event in carcinogenesis. In this study, we examined and compared the methylation levels of LINE-1 between the early and late passages of DPSCs using combined bisulfite restriction analysis. DPSCs showed the morphological change and lost methylation level of LINE-1 during expansion. DPSCs in late passage have lower level of LINE-1 methylation than the early passage but with no statistical significant difference. Further study about epigenetic and malignant transformation of DPSCs is still recommended and required for the secure application of these cells.

Keywords: LINE-1, Methylation, Dental pulp stem cell, Passages

Received Date: APR 27, 2017 **Accepted Date:** JUL 17, 2017

doi: 10.14456/jdat.2017.20

Correspondence to:

Keskanya Subbalekha. Department of Oral and Maxillofacial Surgery, Faculty of Dentistry, Chulalongkorn University. 34 Henri-Dunant Road, Wangmai, Pathumwan, Bangkok 10330 Thailand Tel. 02-218-8581 E-mail: skeskanya@gmail.com

Introduction

Mesenchymal stem cells play an important part in tissue engineering and stem cell-based therapies for repairing and regenerating damaged tissue due to their potential in self-renewal and differentiation into many cell type.[1-3] Human dental pulp stem cell (DPSC), isolated from pulpal tissue inside the tooth chamber, is a practical source of stem cells in because of its easy access and low morbidity.[4] They can be harvested from discarded tooth including wisdom tooth. DPSCs have a high proliferation rate and display typical fibroblast-like morphology with high clonogenic activity similar to the mesenchymal stem cells from human bone-marrow.[5] They can differentiate into several cell types, such as neurons, adipocytes, osteoblasts, chondrocytes, keratocytes, and insulin-producing cells;[6-12] thus, they become a future promising tool in the large field of regenerative medicine.

A crucial problem for the application of DPSC is the small amount of cells that can be isolated; therefore, cell expansion *in vitro* is required. However, long-term culture leads to the changes in morphological, proliferation potential and differentiation potential of DPSCs.[13,14] Moreover, the possible accumulation of mutation that can bring the malignant transformation should be concerned.

Global loss of methylation level is an evident process that occur during tumor progression.[15] The DNA methylation is a major epigenetic regulatory process which methyl groups are covalently added to base cytosine in the DNA strand.[16,17] It is an efficient repressor of transcriptional activity as the methyl groups obviate the binding of transcription factors and found related with many regulation of biological processes including proper development,[18-20] parental genomic imprinting,[21,22] genomic stability, long-term gene silencing,[23,24] and gene expression that regulate cell function and differentiatio.[25] More than one-third of DNA methylation occurs in the repetitive elements.[26,27] Interspersed repetitive sequences are the major contributor to human genome, accounting for 45 percentages of human DNA,[28,29] and can be classified by size and the association of transposable elements. LINE-1 is the most abundant long interspersed elements (LINEs) and comprise approximately17 percentages of human genome. In addition, LINE-1 hypomethylation occur as an early event in carcinogenesis.[15] Decreasing in methylation level of LINE-1 is found relating with many types cancer, for example head and neck cancer, lung cancer, colon cancer, hepatic cancer, prostate cancer and bladder cancer.

In this study, we propose to preliminarily investigate and compare the LINE-1 methylation levels in different passage of DPSCs. The findings from this study will help us to initially understand more about epigenetic event of dental pulp stem cell and will lead to the appropriate further study. All aspects of biological mechanisms involving DPSCs culture should be clearly understood for the efficiently and securely application of DPSCs in the future.

Objectives

The objective of this pilot study is to preliminarily examine and compare the methylation levels of LINE-1 between the early and late passages of human dental pulp stem cells.

Materials and Methods

1. Sample and DNA Extraction

The protocol for the isolation of dental pulp stem cells was approved by the Ethical Committee, Faculty of Dentistry, Chulalongkorn University. Five non-pathological third molars from healthy adult subjects who underwent surgical extraction at Department of

Oral and Maxillofacial surgery, Faculty of Dentistry, were used for the isolation of DPSCs in this pilot study. Dental pulp tissue was explanted and plated in 60 mm. culture dish and maintained in 3 ml. of Dulbecco's modified Eagle's medium (DMEM; Gibco) containing 10 % fetal bovine serum (FBS, Gibco), 2 mM L-glutamine 100 (Gibco), 100 U/ml penicillin, 100 ug/ml streptomycin and 5 ug/ml amphotericin B (Gibco) in 100 % humidity, 37°C and 5 % carbon dioxide under sterile condition. Medium was changed every 48 hours. The cells were subcultured to 3 plates at a 1:3 ratio after reaching 80 % confluence.

DPSCs underwent standard passaging procedure until the cell proliferation rate was extremely decreased. DPSCs were observed their morphology under light microscope and collected for DNA extraction in order to analyze the methylation level of LINE-1. Collected cells were subject to lysis using proteinase-K lysis buffer at 50°C for 48 h. The DNA was then isolated by standard phenol-chloroform extraction.

2. Bisufite Modification

After DNA concentrations were determined with a spectrophotometer, bisulfite modification of the DNA samples was performed using an EZ DNA methylation kit (Zymo Research, Orange, CA, USA). Briefly, bisulfite treatment converts unmethylated cytosine to uracil, whereas the methylated cytosine remain unchanged.

3. The quantitative combined bisulfite restriction analysis-LINE1 (qCOBRA-LINE1)

Briefly, one microliters of bisulfite DNA was annealed with primers for COBRA LINE-1, 5-GTTAAAGAAAGGGGTGAYGGT -3 and 5-AATACRCCRTTTCTTAAACRA TCTA-3 at 55°C and amplified for 30 cycles.

The LINE-1 amplicons after amplification (92 bp length) were digested in 10 µL volumes with 2 U of TasI in 1x TaqI buffer (MBI Fermentas, Burlington, Canada) and incubated overnight at 65°C. The DNA fragments were then electrophoresed in 8 % polyacrylamide gel and stained with the SYBR green (Gelstar, Lonza, Rockland, ME, USA), resulting in separated five bands of DNA. The intensity of the DNA fragments was measured using a Phosphoimager with Image Quant software (Molecular Dynamics, GE Healthcare, Slough, UK). DNA templates from HeLa cell lines were used as controls for normalization of the inter-assay variation between each experiment. The 5 bands on the electrophoresis of LINE-1 are 92 bp, 60 bp, 50 bp, 42 bp, and 32 bp (Fig. 1).

4. LINE-1 Methylation Analysis

An illustration of the qCOBRA-LINE1 technique and an example of gel electrophoresis are shown in Figure 1. The COBRA LINE-1 loci were categorized into four groups: two unmethylated CpGs (uCuC); two methylated CpGs (mCmC); 5'-methylated and 3'-unmethylated CpGs (mCuC); and 5'-unmethylated and 3'-methylated CpGs (uCmC), based on the methylation status of 2 CpG dinucleotides in the 5' and 3' regions of the LINE-1 sequence. The DNA fragments derived from enzymatic digestion of the COBRA-LINE1 products were separated into 5 fragments of 92, 60, 50, 42, and 32 bp, which represented different methylation states. The number of CpG dinucleotides was calculated by dividing the intensity of each band by the number of double-stranded bp of DNA sequence as follows: A=intensity of the 92-bp fragment divided by 92; B=intensity of the 60-bp fragment divided by 56; C=intensity of the 50-bp fragment divided by 50; D=intensity of the 42-bp fragment divided by 40; E=intensity of the 32-bp fragment divided by 32; and F=((D+E)-(B+C))/2. The LINE-1 methylation levels were calculated with the following formula: LINE-1 methylation level percentage (% mC) = 100×(2C+A+F) / (2C+2A+2F+2B).

The statistical analysis was performed using SPSS software for Windows version 22.0 (SPSS Inc., Chicago, IL). A signed rank test was performed to test the difference between methylation levels of DPSCs in early and late passage. A P value<0.05 was considered statistically significant.

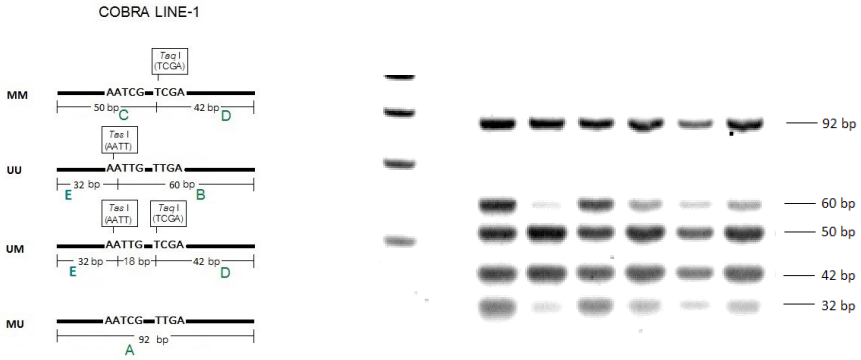

Figure 1 *The illustration of the qCOBRA-LINE-1 technique and the example of gel electrophoresis.*

Results

1. Morphological Observation

In the early passage, 4th passage of DPSCs showed a fibroblast-like morphology, single monolayer with a well-spread morphology attached to the culture dish (Fig. 2A). The morphology of DPSCs has gradually changed over time that we subculture *in vitro.* In the late passage, DPSCs at 15th passage showed enlarged cell size, increased cell secretion, increased nuclear/ cytoplasm ratio and more cytoplasmic processes (Fig. 2B).

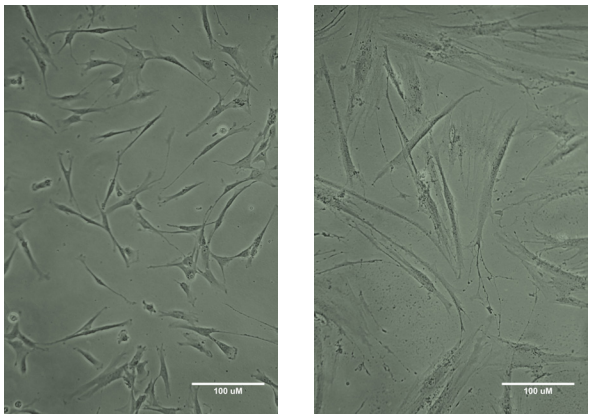

Figure 2 *The illustrations of dental pulp stem cells morphology in early and late passages. (A); DPSCs at 4th passage, (B); DPSCs at 15th passage under light microscope (original magnification, x100). DPSCs in late passage showed enlarged cell size and more cytoplasmic processes. White arrow showed the scattered cell secretion that was obviously seen on the culture dish background in late passage.*

2. Methylation levels of LINE-1

Figure 3 showed the methylation levels (% mC) of DPSCs in 4th (early) and 15th (late) passages. The LINE-1 methylation levels of sample 1, 2, 3, 4, and 5 are 57.229, 54.245, 55.604, 73.819 and 69.276 in 4th passage, while 57.509, 52.437, 53.119, 67.126, and 67.880 repectively in 15th passage. Four out of five DPSCs samples had lower level of LINE-1 methylation at 15th passage than the 4th passage, whereas one sample showed a very slightly higher level of LINE-1 methylation in 5th passage. The average LINE-1 methylation level is 62.035 ± 8.895 in 4th passage of DPSCs and 59.614 ± 7.465 in 15th passage of DPSCs. However, there was no statistically significant difference in LINE-1 methylation level between the early and late passage of DPSCs (p = .08).

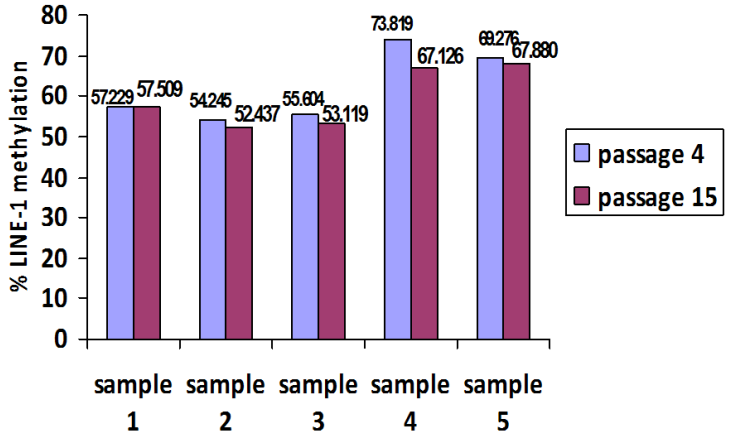

Figure 3 *The methylation levels (% mC) of DPSCs at 4th and 15th passages. Most of DPSCs samples had lower level of LINE-1 methylation at 15th passage than the 4th passage.*

Discussion

The limitations of this study are the sample size and the number of passages. More passage number is needed in the further study. The microbial contamination was one of the major hindrances in long-term cell cultivation that could limit the number of passage. Moreover, longer time was required for the more number of passages. DPSCs in early passage took 3-5 days to reach the confluence, while it took more than 2 weeks to reach the confluence in the late passage. In this study, two samples of DPSCs can reach more than passage 18 but we assorted the DPSCs at 15th passage as the late passage DPSCs since all samples showed the obvious alteration in morphology and could reach this passage number. However, passage number is not the exact cellular age. It simply refers to the number of times the cells have been subcultured. The inoculation densities and recoveries should be concerned.

The declination of LINE-1 methylation level of DPSCs at 15th passage, compared to 4th passage was rather small to detect the statistical significant difference, so the larger sample size is needed.

Our study reveals that DPSCs were capable of long-term cultivation under the *in vitro* conditions we provided without changing their viability but losing their proliferation rate. The morphologic alteration of DPSCs are found during *in vitro* expansion after some passages, such as, alteration in cell shape, enlarged cell size, increased cell secretion and nuclear/cytoplasm ratio. These findings are in agreement with the study of Liu *et al.*[14] who also reported the sequential loss of reprogramming markers Oct-4, Sox2, and c-Myc in the nucleus during dental pulp cell cultures.

LINE-1 (Long interspersed nuclear element-1) retrotransposons are the mobile elements or jumping genes that comprise about 17 percent of human DNA. They multiply themselves throughout the genome. Their methylation statuses are associated with cancer initiation and progression. Decreasing in methylation level of LINE-1 is found relating with many types cancer[30-35] and other pathologic conditions.[36,37] In this study, although we demonstrated that DPSCs at 15th passage lost LINE-1 methylation, we cannot consider their tumorigenesis because they also lost their proliferation rate. The further study about epigenetic mechanisms and malignant transformation of DPSCs is still recommended and required for the secure application of these cells.

Conclusion

Dental pulp stem cells slightly lost their methylation level of LINE-1 during cultured passage *in vitro* but with no statistically significant difference. The larger sample size and more passage number should be recruited in further study.

References

1. Bianco P, Robey PG. Stem cells in tissue engineering. ***Nature*** 2001;414:118-21.

2. Bulgin D, Hodzic E, Komljenovic-Blitva D. Advanced and Prospective Technologies for Potential Use in Craniofacial Tissues Regeneration by Stem Cells and Growth Factors. ***J Craniofac Surg*** 2011;22:342-8.

3. Nguyen TT, Mui B, Mehrabzadeh M, Chea Y, Chaudhry Z, Chaudhry K, *et al.* Regeneration of tissues of the oral complex: current clinical trends and research advances. ***J Can Dent Assoc*** 2013;79:d1.

4. Fawzy El-Sayed KM, Dörfer C, Fändrich F, Gieseler F, Moustafa MH, Ungefroren H. Adult mesenchymal stem cells explored in the dental field. ***Adv Biochem Eng Biotechnol*** 2013;130:89-103.

5. Kadar K, Kiraly M, Porcsalmy B, Molnar B, Racz GZ, Blazsek J, *et al.* Differentiation potential of stem cells from human dental origin - promise for tissue engineering. ***J Physiol Pharmacol*** 2009;60:167-75.

6. Sawangmake C, Nowwarote N, Pavasant P, Chansiripornchai P, Osathanon T. A feasibility study of an in vitro differentiation potential toward insulin-producing cells by dental tissue-derived mesenchymal stem cells. ***Biochem Biophys Res Commun*** 2014;452:581-7.

7. Iohara K, Zheng L, Ito M, Tomokiyo A, Matsushita K, Nakashima M. Side population cells isolated from porcine dental pulp tissue with self-renewal and multipotency for dentinogenesis, chondrogenesis, adipogenesis, and neurogenesis. ***Stem cells*** 2006;24:2493-503.

8. Jo YY, Lee HJ, Kook SY, Choung HW, Park JY, Chung JH, *et al.* Isolation and characterization of postnatal stem cells from human dental tissues. ***Tissue Eng*** 2007;13:767-73.

9. Ueno A, Yamashita K, Miyoshi K, Horiguchi T, Ruspita I, Abe K, *et al.* Soluble matrix from osteoblastic cells induces mineralization by dental pulp cells. The journal of medical investigation. ***J Med Invest*** 2006;53:297-302.

10. Hosoya A, Nakamura H, Ninomiya T, Hoshi K, Yoshiba K, Yoshiba N, *et al.* Hard tissue formation in subcutaneously transplanted rat dental pulp. ***J Dent Res*** 2007;86:469-74.

11. Otaki S, Ueshima S, Shiraishi K, Sugiyama K, Hamada S, Yorimoto M, *et al.* Mesenchymal progenitor cells in adult human dental pulp and their ability to form bone when transplanted into immunocompromised mice. ***Cell Biol Int*** 2007;31:1191-7.

12. Syed-Picard FN, Du Y, Lathrop KL, Mann MM, Funderburgh ML, Funderburgh JL. Dental pulp stem cells: a new cellular resource for corneal stromal regeneration. ***Stem Cells Transl Med*** 2015;4:276-85.

13. Mehrazarin S, Oh JE, Chung CL, Chen W, Kim RH, Shi S, *et al.* Impaired odontogenic differentiation of senescent dental mesenchymal stem cells is associated with loss of Bmi-1 expression. ***J Endod*** 2011;37:662-6.

14. Liu L, Wei X, Ling J, Wu L, Xiao Y. Expression pattern of Oct-4, Sox2, and c-Myc in the primary culture of human dental pulp derived cells. ***J Endod*** 2011;37:466-72.

15. Mutirangura A. Quantitative PCR analysis for methylation level of genome: clinical implications in cancer. Asian Biomed 2007;1:121-8.

16. Jones PA, Takai D. The role of DNA methylation in mammalian epigenetics. ***Science*** 2001;293:1068-70.

17. Dean W, Lucifero D, Santos F. DNA methylation in mammalian development and disease. ***Birth Defects Res C Embryo Today*** 2005;75:98-111.

18. Santos F, Dean W. Epigenetic reprogramming during early development in mammals. ***Reproduction*** 2004;127:643-51.

19. Waterland RA, Kellermayer R, Rached MT, Tatevian N, Gomes MV, Zhang J, *et al.* Epigenomic profiling indicates

a role for DNA methylation in early postnatal liver development. Hum Mol Genet 2009;18:3026-38.
20. Razin A, Shemer R. DNA methylation in early development. ***Hum Mol Gene***t 1995;4:1751-5.
21. Reik W, Howlett SK, Surani MA. Imprinting by DNA methylation: from transgenes to endogenous gene sequences. ***Dev Suppl*** 1990:99-106.
22. Sapienza C, Peterson AC, Rossant J, Balling R. Degree of methylation of transgenes is dependent on gamete of origin. ***Nature*** 1987;328:251-4.
23. Klose RJ, Bird AP. Genomic DNA methylation: the mark and its mediators. ***Trends Biochem Sci*** 2006;31:89-97.
24. Hoffman AR, Hu JF. Directing DNA methylation to inhibit gene expression. ***Cell Mol Neurobiol*** 2006;26:425-38.
25. Bird A. DNA methylation patterns and epigenetic memory. ***Genes Dev*** 2002;16:6-21.
26. Kochanek S, Renz D, Doerfler W. DNA methylation in the Alu sequences of diploid and haploid primary human cells. ***EMBO J*** 1993;12:1141-51.
27. Schmid CW. Does SINE evolution preclude Alu function? ***Nucleic Acids Res*** 1998;26:4541-50.
28. Mills RE, Bennett EA, Iskow RC, Devine SE. Which transposable elements are active in the human genome? ***Trends Genet*** 2007;23:183-91.
29. Pheasant M, Mattick JS. Raising the estimate of functional human sequences. ***Genome Res*** 2007;17:1245-53.
30. Sirivanichsuntorn P, Keelawat S, Danuthai K, Mutirangura A, Subbalekha K, Kitkumthorn N. LINE-1 and Alu hypomethylation in mucoepidermoid carcinoma. ***BMC Clin Pathol*** 2013;13:10.
31. Kitkumthorn N, Keelawat S, Rattanatanyong P, Mutirangura A. LINE-1 and Alu methylation patterns in lymph node metastases of head and neck cancers. ***Asian Pac J Cancer Prev*** 2012;13:4469-75.
32. Tangkijvanich P, Hourpai N, Rattanatanyong P, Wisedopas N, Mahachai V, Mutirangura A. Serum LINE-1 hypomethy lation as a potential prognostic marker for hepatocellular carcinoma. ***Clin Chim Acta*** 2007;379:127-33.
33. Shuangshoti S, Hourpai N, Pumsuk U, Mutirangura A. Line-1 hypomethylation in multistage carcinogenesis of the uterine cervix. ***Asian Pac J Cancer Prev*** 2007;8:307-9.
34. Pattamadilok J, Huapai N, Rattanatanyong P, Vasurattana A, Triratanachat S, Tresukosol D, *et al.* LINE-1 hypomethylation level as a potential prognostic factor for epithelial ovarian cancer. ***International Int J Gynecol Cancer*** 2008;18:711-7.
35. Cash HL, Tao L, Yuan JM, Marsit CJ, Houseman EA, Xiang YB, *et al.* LINE-1 hypomethylation is associated with bladder cancer risk among nonsmoking Chinese. ***Int J Cancer*** 2012;130:1151-9.
36. Nakkuntod J, Avihingsanon Y, Mutirangura A, Hirankarn N. Hypomethylation of LINE-1 but not Alu in lymphocyte subsets of systemic lupus erythematosus patients. ***Clin Chim Acta*** 2011;412:1457-61.
37. Wei L, Liu S, Su Z, Cheng R, Bai X, Li X. LINE-1 hypomethylation is associated with the risk of coronary heart disease in Chinese population. ***Arq Bras Cardiol*** 2014;102:481-8.

Original Article

Diameter and Density of Dentinal Tubule in Human Primary Teeth

Jittikarn Sikanta[1], Sitthichai Wanachantararak[2] and Varisara Sirimaharaj[1]

[1]Department of Orthodontics and Pediatric Dentistry, Faculty of Dentistry, Chiang Mai University, Chiang Mai
[2]Department of Oral Biology and Diagnosis Sciences, Faculty of Dentistry, Chiang Mai University, Chiang Mai

Abstract

The aim of this study was to investigate dentinal tubule diameter and dentinal tubule density in primary teeth and to compare those values between each tooth type at electron microscope level. Thirty freshly extracted primary teeth were included in this experiment and categorized into six groups according to their tooth types and dental arches. All samples were first sectioned longitudinally, and then sectioned perpendicular to the direction of dentinal tubules which directed from the pulpal horn at three levels represented outer, middle and inner dentin. The result revealed that the diameter and density of dentinal tubule increased from outer dentin toward the pulp. The mean±SD of tubule diameter was largest in maxillary molar (1.44±0.24µm) and smallest in mandibular incisor (1.15±0.18µm). The density of dentinal tubules was greatest in mandibular incisor (58,080.33 tubules/mm^2) and lowest in maxillary molar (27,476.26 tubules/mm^2). The diameter of dentinal tubules tended to increase from anterior to posterior teeth, while, conversely, tubule density decreased from anterior to posterior teeth.

Keywords: Dentinal tubule, Primary teeth, Tubule density, Tubule diameter

Received Date: APR 27, 2017 **Accepted Date:** JUL 17, 2017
doi: 10.14456/jdat.2017.21

Correspondence to:
Sitthichai Wanachantararak. Department of Oral Biology and Oral Diagnostic Sciences, Faculty of Dentistry, Chiang Mai University, Chiang Mai 50200 Thailand Tel.: 081-671-4004 E-mail: sitthichai.w@cmu.ac.th

Introduction

The tubular structure of dentin makes it unique from other hard tissue in the body. The tubules are not only used for transportation of mineral salt to deposit at the calcified front at the mineral wall but also play an important role in transferring stimuli and irritants to nerve terminal at the surface of the dental pulp. After loss its coverage, dentin became permeable to environment, opened for invasion of bacteria and its toxin while it was having continuous outward flow to counter balance.[1]

The model composite restoration utilizes the porous property of dentin by desiring an adhesive to form a mechanical lock with decalcified dentine on etched surface and inside the tubule forming hybrid layer and resin tags. The density and diameter of dentinal tubules have strong relation with bond strength of dental adhesive.[2-4]

There is a possibility that primary teeth have different properties from permanent teeth. However, most of bond strength studies were experimented on permanent human teeth or even animal teeth. While primary teeth have many factors, that should be taking into account before making any restoration. Only few reports published on the diameter and density of dentinal tubules. Therefore, this study aimed to investigate the properties of primary dentin by measuring tubule diameter and the number of tubules per area at different depth of coronal dentin and comparing those values between each tooth type at electron microscope level.

Materials and Methods

This study has been approved by The Human Experimentation Committee of the Faculty of Dentistry, Chiang Mai University. All teeth specimens were collected under consent of participants and their parents.

Thirty freshly extracted prolonged primary teeth from 4-14-year-old children were included in this study. The tooth specimens must be intact or have one small proximal carious lesion extend no further than one-third of the dentin thickness.

The specimens were rinsed in running tap water immediately after extraction and stored at 4°C in normal saline solution with 0.1 % thymol until used.

All samples were divided into 6 groups according to their tooth types and dental arches; maxillary incisors (n=6), maxillary canines (n=4), maxillary molars (n=5), mandibular incisors (n=7), mandibular canines (n=3), and mandibular molars (n=5).

The roots were cut off at the level of 1 mm apical to cementoenamel junction (CEJ) by using a cylinder diamond bur (Intensive®, Swiss Dental Product, Switzerland) attached on an air rotor handpiece with water spray cooling. The remaining pulp tissues were removed by using endodontic barb broach.

Tooth preparation procedure for incisors and canines were similar. Crowns were cut longitudinally in buccolingual direction through the middle part of the crowns in order to gain access to the most straight dentinal tubules running from the pulpal horn to the dentinoenamel junction (DEJ) as shown in Fig. 1A.

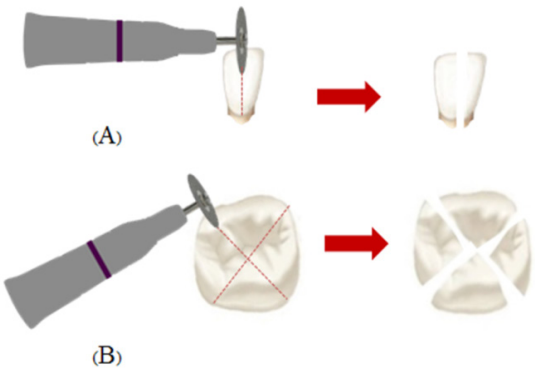

Figure 1 *Tooth specimen preparation in anterior tooth (A) and posterior tooth (B)*

In order to measure the most straight dentinal tubules which run from the tip of each pulpal horn to the tip of the cusp in posterior teeth, crown of posterior tooth having three to four pulpal horns were cut longitudinally in two directions from mesiobuccal line angle to distolingual line angle and from distobuccal line angle to mesiolingual line angle (Fig. 1B).

In addition, all specimens were sectioned perpendicular to dentinal tubules at 1/4, 1/2, 3/4 of its thickness to represent outer, middle and inner dentin, respectively (Fig. 2).

The cut tooth surface were cleaned by stored the whole specimen in 5.25 % sodium hypochlorite solution for 12 hours then placed into an ultrasonic cleaner for 10 minutes. The samples were left dried at room temperature in clean ventilation storage for 24 hours. The specimens were attached to the stub with conductive adhesive and coated with gold-palladium under vacuum in sputter coater equipment (JEOL® JFC1200 Fine Coater; JEOL, Tokyo, Japan).

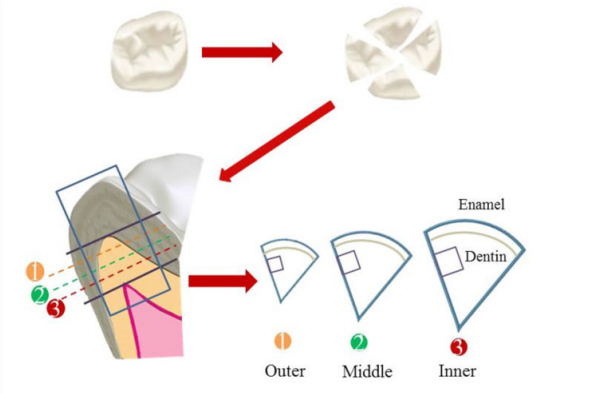

Figure 2 *Dentinal tubules over the pulpal horn at each dentin level were examined by SEM.*

The sample was examined under scanning electron microscope (SEM) (JEOL® JSM 6610LV, JEOL Ltd., Tokyo, Japan) at 15kV. The SEM images of the same dentinal tubules over the pulp horn at each level were captured at magnification of x3000 into the digital image files for further analysis.

The tubule diameter was measured by using the image analysis software, Image J (NIH, USA) from the digital images compare to the calibration bar. The formula proposed by Forssell-Ahlberg et al. (1975)[5] was used to calculate the number of dentinal tubules/mm^2.

$$X = \frac{10^6}{(l/i)^2} \cdot n$$

Where X = number of tubule /mm^2

l = length of side of photomicrograph (μm)

i = magnification

n = number of dentinal tubules on the photomicrograph

Every dentinal tubules presented in the SEM images was counted excluding those tubules that smaller than semi-circle at the edge of images and those tubules with branched at the outer dentin. To minimize the error results from sectioning, the shortest part of ovoid tubule was used to represent the diameter of that tubule.

Seventy-seven dentinal tubules which was the least counted number of the tubules on the SEM images at x3000 magnification from each group was selected to statistically compare all samples groups. The results were analyzed by using statistical analysis program (SPSS version 22.0, SPSS Science, Chicago, USA). The difference in means was analyzed by two-way ANOVA and Tukey's test pairwise comparison (p<0.05).

Results

Representative SEM images of dentinal tubules in primary teeth at different dentin depths were shown in Fig. 3.

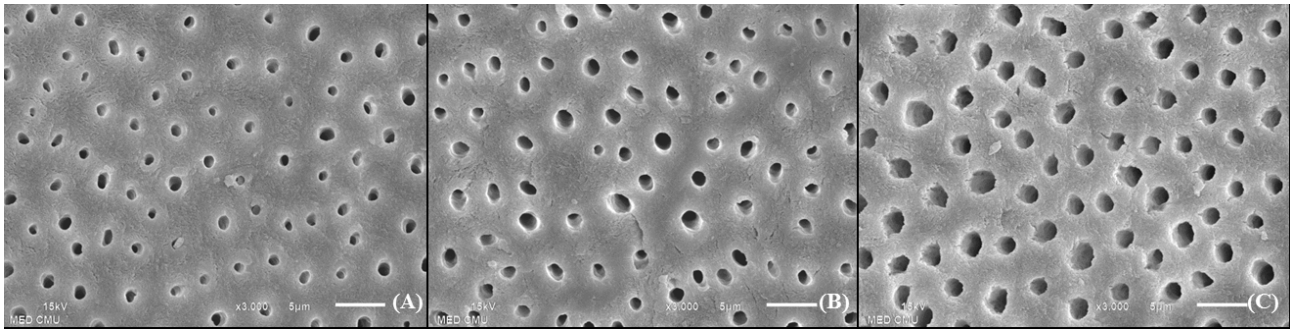

Figure 3 *Samples of SEM images of dentinal tubules in outer (A), middle (B), and inner (C) dentin of primary maxillary lateral incisor*

The mean±SD value of tubule diameter in each tooth type was shown in Table 1. The diameter of dentinal tubule increased significantly ($p<0.05$) from outer dentin toward the pulp in all tooth type. Overall, the diameter of dentinal tubules in inner dentin were larger than the middle dentin 6.72-22.02 % and larger than the outer dentin 23.33-55.96 %. Regarding tooth types, Molars had significant larger tubule compared to mandibular incisors and canines. The largest mean tubule diameter was maxillary molar and the smallest was in mandibular incisor.

Table 1 *Dentinal tubule diameter by tooth types at different depth*

Tooth type	Mean±SD of tubule diameter (μm)			
	Outer	Middle	Inner	Average
Maxillary incisor (n=6)	1.09±0.04[a]	1.33±0.04[b]	1.70±0.04[c]	1.37±0.25
Maxillary canine (n=4)	1.19±0.36[a]	1.27±0.13[b]	1.59±0.16[c]	1.35±0.30
Maxillary molar (n=5)	1.24±0.17[a]	1.37±0.11[b]	1.69±0.14[c]	1.44±0.24
Mandibular incisor (n=7)	0.96±0.04[a]	1.09±0.02[b]	1.39±0.05[c]	1.15±0.18
Mandibular canine (n=3)	1.20±0.16[a]	1.36±0.13[b]	1.48±0.17[c]	1.34±0.19
Mandibular molar (n=5)	1.19±0.15[a]	1.42±0.14[b]	1.67±0.10[c]	1.43±0.24

**The different superscript character (a,b,c) in the same row indicates significant different at p<0.05 when analyses by two-way ANOVA and Tukey's test pairwise comparison.*

The result of tubule density or the number of tubules per unit area was shown in Fig. 4. Mandibular incisor had the highest average dentinal tubules density while maxillary molar had the lowest number. The range of tubular density at outer dentin was 19,800.51-50,112.40 tubules/mm^2 and at the inner dentin was 35,787.59-64,412.77 tubules/mm^2. The tubular tended to increase from outer to inner layer of dentin in all tooth type.

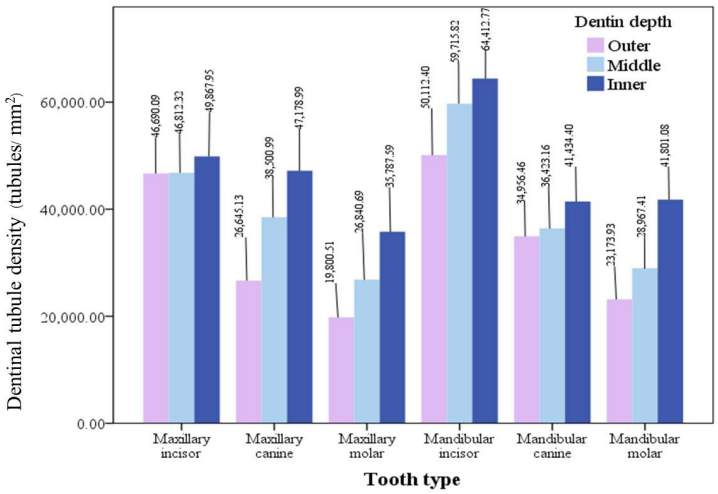

Figure 4 *Dentinal tubule density at different dentin depth in each tooth type (tubules/mm2)*

Discussion

In general, the diameter of dentinal tubule obtained from this study was relatively larger than other studies[3,6,10] even though this study used no acid or chelating agent which might enlarge the size by dissolving mineral content of peritubular dentin.

In more detail, our tubule diameter of primary incisors was larger than the result reported by Costa *et al.*, 2002.[6] At the middle of dentin, the mean tubule diameter of this study was 1.33 μm in maxillary incisor and 1.09 μm in mandibular incisor while of those study was 1.05 μm in general. However, the mean tubular diameter in canine was smaller than the result obtained by Sumikawa *et al.*, (1999).[7]

Schilke and colleague (2000)[8] and Lenzi and colleague (2013)[9] reported larger dentinal tubule in primary molars at middle and inner dentin compared to this present study. However, the opposite results Koutsi *et al.*, 1994 and Ruschel *et al.*, 2012 reported smaller dentinal tubule (0.96-1.29 μm, 0.794-1.00 μm, respectively) compare with this study.[3,10]

The result from this study supported the fact that the number of dentinal tubules per unit area was increasing with depth. The outer dentin had less number of dentinal tubules per unit area than the middle and inner dentin.

The tubule density of incisors and canines found in this study (Fig. 4) were greater than the founding of Costa *et al.*, 2002 (9,641-23,114 tubules/mm^2)[6] and Sumikawa *et al.*, 1999 (approximately 26,000-35,000 tubules/mm^2)[7] respectively. The tubule density of molars found in this study (Fig. 4) were greater than the results from other investigations by Koutsi *et al.*, 1994 (17,433-26,391 tubules/mm^2)[3], Schilke *et al.*, 2000 (18,243-24,162 tubules/mm^2)[8], and Ruschel *et al.*, 2012 (17,997.60-25,211.32 tubules/mm^2)[10] while lower than Lenzi and colleague, 2013 (85,541-171,510 tubules/mm^2) which included canaliculi in their study.[9]

The different area of investigation might affect both diameter and density of the dentinal tubules.[11] Focusing in measuring the straight dentinal tubules travelling from the pulpal horn to either the tip of incisor or the tip of the cusp could be the reason for the higher number of tubules/mm2 in our study compared to other studies.

The average and standard deviation of remaining dentin thickness from all tooth specimens in this study

was 2.35±0.23 mm. The SD value indicated that the collected specimen had small difference in tooth age when it had been extracted.

The difference in diameter and density of dentinal tubules in each tooth types and the depth of dentin will determine the hydraulic conductance value of dentin which allows dentinal fluid to flow in and out at ease. The result from the present study and from previous studies suggested that the increase of diameter density of dentinal tubules related to higher hydraulic conductance of dentin.[4,14-15]

The dentinal tubule diameter and density over the pulpal horn increased significantly among 3 levels of dentin. The results can be explained by the convergence of tubules as they approach the pulp.[9,12-13]

The bond strength studies were mainly experimented on permanent human teeth or animal teeth and the result was implied to use for primary human teeth which may have different in morphology and properties.[16,17]

Our studies provided basic knowledge of dentin characteristic and properties in primary teeth. However, percentage of area which occupied by dentinal tubules and peritubular dentin would be investigated in future.

Conclusion

The diameter and the density of dentinal tubules increased from outer dentin toward the pulp in all tooth type of primary teeth.

The diameter of dentinal tubules in primary teeth tended to increase from anterior to posterior teeth, in opposite, tubule density decreased from anterior to posterior teeth.

Acknowledgement

This research was supported by Faculty of Dentistry, Chiang Mai University. We are thankful for Ms. Thippawan Yasanga for her recommendation in electron microscope using and Dr. Thanapat Sastraruji for his assistance in statistical analysis.

References

1. Pashley DH. Dentin permeability, dentin sensitivity, and treatment through tubule occlusion. ***J Endod*** 1986;12:465-74.

2. Pashley DH, Ciucchi B, Sano H, Horner JA. Permeability of dentin to adhesive agents. ***Quintessence Int*** 1993; 24:618-31.

3. Koutsi V, Noonan R, Horner J, Simpson M, Matthews W, Pashley DH. The effect of dentin depth on the permeability and ultrastructure of primary molars. ***Pediatr Dent*** 1994; 16:29-35.

4. Jariyanuwat T, Sirimaharaj V, Wanachantararak S. Effect of different intrapulpal pressure simulation on bond strength and resin tags length in primary incisors. ***M Dent J*** 2016;36:99-105.

5. Forssell-Ahlberg K, Brannström M, Edwall L. The diameter and number of dentinal tubules in rat, cat, dog and monkey: a comparative scanning electron microscopic study. ***Acta Odontol Scand*** 1975;33:243-50.

6. Costa L, Watanabe IS, Kronka MC. Coronal dentinal tubules of non-erupted deciduous incisors. ***Pesqui Odontol Bras*** 2002;16:12-7.

7. Sumikawa DA, Marshall G, Gee L, Marshall S. Microstructure of primary tooth dentin. ***Pediatr Dent*** 1999;21:439-44.

8. Schilke R, Lisson JA, Bauß O, Geurtsen W. Comparison of the number and diameter of dentinal tubules in human and bovine dentine by scanning electron microscopic investigation. ***Arch Oral Biol*** 2000;45:355-61.

9. Lenzi TL, Camila de Almeida BG, Arana-Chavez VE, Raggio DP. Tubule density and diameter in coronal dentin from primary and permanent human teeth. ***Microsc Microanal*** 2013;19:1445-9.

10. Ruschel HC, Chevitarese O. Density and diameter of dentinal tubules of first and second primary human molars-comparative scanning electron microscopy study. ***J Clin Pediat Dent*** 2002;26:297-304.

11. Mjör I, Nordahl I. The density and branching of dentinal tubules in human teeth. ***Arch Oral Biol*** 1996;41:401-12.
12. Marshall GW, Marshall SJ, Kinney JH, Balooch M. The dentin substrate: structure and properties related to bonding. ***J Dent*** 1997;25:441-58.
13. Carrigan PJ, Morse DR, Furst ML, Sinai IH. A scanning electron microscopic evaluation of human dentinal tubules according to age and location. ***J Endod*** 1984; 10:359-63.
14. Rangcharoen M. The Use of Replica Technique to study Dentin Pemeability in Primary teeth (Master of Science in Dentistry). Orthodontics and Pediatric Dentistry, Faculty of Dentistry. Chiang Mai: Chiang Mai University; 2011.
15. Akkho P. A comparison of microtensile bond strength of different adhesive system on primary incisors under simulated intrapulpal pressure (Master of Science in Dentistry). Orthodontics and Pediatric Dentistry, Faculty of Dentistry. Chiang Mai: Chiang Mai University; 2014.
16. Salarna FS, Tao L. Comparison of Gluma® bond strength to primary vs. permanent teeth. ***Pediatr Dent*** 1991;13:163-66.
17. Bordin-Aykroyd S, Sefton J, Davies EH, *In vitro* bond strengths of three current dentin adhesives to primary and permanent teeth. ***Dent Mater*** 1992;8:74-8.

Original Article

Association between Stress and Periodontitis among Thai Elderly

Rajda Noisombut[2], Suntra Tuntianunanon[1], Suparat Ketchareonkhun[1], Irada Advichai[1] and Waranuch Pitiphat[2]

[1]Faculty of Dentistry, Khon Kaen University, Khon Kaen

[2]Department of Community Dentistry, Faculty of Dentistry, Khon Kaen University, Khon Kaen

Abstract

The association between periodontal inflammation and stress is still limited to the evidence from Western countries. The aim of this study was to investigate whether stress was associated with periodontitis among Thai elderly. The cross-sectional study was conducted on 179 elderly aged 60 and over in Khon Kaen, Thailand. A calibrated dentist recorded clinical attachment levels (CAL) using the random half-mouth six-sites per tooth protocol. A face-to-face interview assessed stress level using Self-analyzed and Self-evaluated Stress Test (SSST). A structured questionnaire was used to obtain the information on demographics, medical history, and oral health behaviors. Logistic regression analysis was used to evaluate the association between periodontitis and stress while controlling for possible confounders. The result indicated that stress was associated with periodontitis (tertiary percentage of CAL≥5 mm) with three times higher odds ratio for those who had stress (OR = 3.0; 95 % confidence interval, 1.3 to 7.0) after controlling for all possible confounders. This study suggests that periodontitis is associated with stress in the elderly.

Keywords: Stress, Periodontitis, Elderly

Received Date: APR 27, 2017 **Accepted Date:** JUL 17, 2017

doi: 10.14456/jdat.2017.22

Correspondence to:

Rajda Noisombut Department of Community Dentistry, Faculty of Dentistry, Khon Kaen University, Muang, Khon Kaen 40002 Thailand

Tel: 043 202405 Fax: 043 202862 Email: rajda_5@hotmail.com

Introduction

Thailand is experiencing an epidemiological transition characterized by a shift from infectious to non-communicable chronic diseases.[1] Systemic diseases and psychological problems due to aging are especially prevalent in old-aged people and adverse life events have a significant impact on psychological stress in the elderly. Moreover, high levels of tooth loss and high prevalence of periodontal disease, indicating poor oral health, are primarily seen in among older people.[2] National oral health survey in 2013 revealed that a subpopulation of 30 % to 89 % of Thai elderly, depending on the measure of disease applied was affected by advanced destructive periodontal disease.[3]

Periodontal disease is a low-grade inflammatory disease caused by specific periopathogenic bacteria.[4] It is known to vary by systemic risk factors, such as diabetes mellitus, smoking, age, and genetic factors. The onset, progress, and severity of the disease are determined by the individual host response.[5]

Since the 1950s, psychological factors have been identified in periodontal disease development[6] and several epidemiological surveys revealed that stress may be a contributing factor of periodontal disease.[7-9] The possible role for an association is supported by some studies,[10, 11] showing that psychosocial conditions may affect the host immune response and cause the individual to be more susceptible to the development of unhealthy conditions including periodontal disease. In addition, stress may also influence a person's behavior and affect on periodontal conditions. Individuals with high stress levels tend to change their habits to be harmful to periodontal health, such as negligent oral hygiene, increased rate of tobacco use, or changes in eating behavior resulting in adverse effects for the function of immunologic system.[9]

Psychological stress has been shown to affect the periodontal status, but the findings are reported only in Western countries. Hence, we conducted a study that aimed to investigate the relationship between stress and periodontitis of the elderly in Khon Kaen, Thailand.

Materials and Methods

This cross-sectional study was carried out among the elderly in Muang District of Khon Kaen province during June-September 2016. The study protocol was approved by Khon Kaen University Ethics Committee for Human Research (HE592183). Eligibility criteria of participants included being 60 years of age or above at the time of interview, and ability to communicate. Exclusion criteria were: (1) having dementia or schizophrenia; (2) having visual impairment, hearing impairment, or deafness; and (3) complete edentulousness. A stratified random sampling was employed to identify participants. Five sub-districts were randomly chosen to represent the central area of Muang District as well as surrounding areas in each direction. Eligible subjects in each sub-district were then randomly selected and invited to meet the investigators at sub-district health center. All participants provided written informed consent before taking part in the study.

Assessment of Stress

Data collection was conducted through face-to-face interview by a trained interviewer. A structured questionnaire was used to obtain the information on demographics, medical history, and oral health behaviors. Stress level was assessed using Self-analyzed and Self-evaluated Stress Test (SSST),[12] which is a standardized tool developed by the Department of Mental Health, Ministry of Public Health, Thailand. The test consists of 20 items measuring the level of perceived stress during the past 2 months. Each item was responded on a 4-point Likert scale (0 = never, 1 = sometimes, 2 = often and 3 = regular). The total score is a sum of all items and interpreted as follows: 0-5 (less stress than a normal

level), 6-17 (normal level of stress), 18-25 (mild level of stress), 26-29 (moderate level of stress) and over 30 (severe stress). Participants with a total score of 18 or above were classified as having stress in this study.

Oral Examinations

Examinations of periodontal status and dental caries were performed at dental clinic in the sub-district health center using mouth mirror and periodontal probe. In two centers without dental clinic, the participants were examined on mobile dental chair under portable halogen light. Periodontal probing depth (PD) and clinical attachment levels (CAL) were examined using the random half-mouth six-sites per tooth protocol.[13] The examiner was the sixth-year dental student who was well-calibrated with a periodontist. Duplicate examination was carried out in 10 percent of the samples. Intra-examiner and inter-examiner reliability of periodontal examination were good with an ICC>0.8 for each. Dental caries was evaluated using the World Health Organization (WHO) criteria, and decayed, missing and filled teeth (DMFT) index was calculated.

Statistical Analysis

Data are expressed as means and standard deviations (SDs) for continuous variables and as frequencies and percentages for categorical variables. Data were analyzed using IBM SPSS software version 19.0 (SPSS, Chicago, IL, USA). Periodontal status was a priori classified in tertiles based on the percentage of sites with CAL≥5 mm. Individuals in the lowest tertile (0 % - 12.4 %) were considered as no to mild periodontitis; those in the middle tertile (12.5 % - 52.7 %) as moderate periodontitis; and those in the highest tertile (≥52.8 %) as severe periodontitis. Additionally, we defined periodontitis as having 30 % of sites with CAL≥5 mm according to Hilgert *et al*[14] in order to compare our results to their report. Logistic regression was performed with enter method to obtain odds ratios (ORs) and 95 % confidence intervals (CIs) of the relationships between moderate to severe periodontitis and associated factors. Potential confounders were included in multivariate analysis if they changed the OR of the association between stress and periodontitis more than 10 percent. Important risk factors for periodontitis including age, gender and smoking were adjusted in the analyses.

Results

Characteristics of the elderly participants

There were 179 elderly subjects participated in this study. The average age (SD) of the participants was 67.1 (5.7) years. The oldest participant was 90 years old. The majority of participants were female (58.1 %), married (64.8 %), having primary school education (62.1 %), and living with family (93.8 %). Almost half (46.4 %) were not working, 41.3 % employed, and the rest were either had own business or worked in agriculture (Table 1). Most participants were non-smoker and non-alcohol drinker. Only 14.5 % were free of systemic diseases. Regarding oral health behaviors, 88.3 % of the participants brushed their teeth daily and 78.8 % used fluoride toothpaste. More than 80 % had previous dental treatment, but only 29.1 % had an annual dental visit (Table 2).

Table 1 *Characteristics of Study Participants (n = 179)*

Characteristic	N (%)
Gender	
- Male	75 (41.9)
- Female	104 (58.1)
Age in years	
- 60-69	131 (73.2)
- 70-79	40 (22.3)
- ≥80	8 (4.5)
Marital status	
- Never married	6 (3.4)
- Married	116 (64.8)
- Widowed	55 (30.7)
- Divorced	2 (1.1)
Education	
- No formal education	46 (25.7)
- Primary school	111 (62.1)
- Secondary school or higher	22 (12.2)
Occupation	
- Not working	83 (46.4)
- Employed	74 (41.3)
- Agriculture	14 (7.8)
- Business	8 (4.5)
Monthly family income in Thai Baht	
- <5,000	73 (40.8)
- 5,001-20,000	87 (48.6)
- >20,000	19 (10.6)
Living arrangement	
- Alone	11 (6.2)
- With children	86 (48.1)
- With spouse	76 (42.5)
- With relatives	6 (3.2)

Table 2 *Health Behavior Information (n = 179)*

Characteristic	N (%)
Current smoking	
- Yes	48 (26.8)
- No	131 (73.2)
Alcohol drinking	
- Yes	60 (33.5)
- No	119 (66.5)
Presence of systemic disease	
- Absent	26 (14.5)
- Cardiovascular disease	60 (37.5)
- Endocrine system disease	58 (36.3)
- Respiratory disease	17 (10.6)
- Other	25 (15.6)
History of dental treatment	
- No previous treatment	32 (17.9)
- Had previous treatments	147 (82.1)
Annual dental visits	
- Yes	52 (29.1)
- No	127 (70.9)
Frequency of tooth brushing	
- Less than once daily	21 (11.7)
- Once daily	64 (35.8)
- At least twice daily	94 (52.5)
Type of toothpaste	
- Fluoride toothpaste	141 (78.8)
- Non-fluoride toothpaste	35 (19.6)
- Tooth powder	3 (1.6)

Stress levels

Based on the SSST, 36.3 % of the elderly were under stressed condition. The majority had a mild level of stress (27.9 %). Only 3.9 % and 4.5 % were affected by moderate and high levels of stress, respectively (Table 3).

Table 3 *Stress levels of the participants based on Self-analyzed and Self-evaluated Stress Test (SSST)*

SSST score	N (%)
No stress	
0-5 (Less than normal level)	25 (14.0)
6-17 (Normal level)	89 (49.7)
With stress	
18-25 (Mild level)	50 (27.9)
26-29 (Moderate level)	7 (3.9)
≥30 (Severe level)	8 (4.5)

Oral health status

The prevalence of caries experience (DMFT>0) among the elderly was 83.8 %, with the average DMFT of 16.0 (SD = 7.3). The average number of teeth with untreated caries was 2.7 (SD = 2.7). Regarding the periodontal status, the participants had an average CAL of 4.1 (SD = 1.5) mm. Most participants (90 %) had at least 1 site with CAL≥5 mm. When the participants were classified based on the percentage of sites with CAL≥5 mm in tertiles, 25.7 % represented no to mild periodontitis, 48.6 % were moderate periodontitis, and 25.7 % were severe periodontitis.

Stress and periodontitis

Table 4 shows the bivariate logistic regression analysis of factors related to moderate to severe periodontitis. Stress was significantly associated with increased odds for moderate to severe periodontitis (OR = 2.8, 95 % CI = 1.4-5.7). The other characteristic that had a significant influence on periodontal status was gender (OR = 2.1, 95 % CI 1.1-4.1 for males).

Table 4 *Factors Associated with Moderate to Severe Periodontitis among Elderly in Khon Kaen Province in Simple Logistic Regression*

Characteristic		Periodontiti		Odds ratio (95 % Confidence Interval)	*P-value*
		No to Mild N (%)	Moderate to severe N (%)		
Gender	Female	42 (40.4)	62 (59.6)	1	
	Male	18 (24.0)	57 (76.0)	**2.1 (1.1-4.1)**	0.02
Age	60-69 years	44 (33.6)	87 (66.4)	1	
	70 years or above	16 (33.3)	32 (66.7)	1.0 (0.5-2.0)	0.98
Marital status	Single (never married, divorced, widowed)	17 (26.9)	46 (73.1)	1.6 (0.8-3.1)	0.17
	Married	43 (37.1)	73 (62.9)	1	
Having formal education	No	15 (32.6)	31(67.4)	1.1 (0.5-2.2)	0.88
	Yes	45 (33.8)	88 (66.2)	1	
Currently working	No	24 (28.9)	59 (71.1)	1.5 (0.8-2.8)	0.23
	Yes	36 (37.5)	60 (62.5)	1	
Monthly family income	≤5,000 Baht	23 (31.5)	50 (68.5)	1.2 (0.6-2.2)	0.64
	>5,000 Baht	37 (34.9)	69 (65.1)	1	
State of living	Alone	2 (18.2)	9 (81.8)	2.4 (0.5-11.3)	0.28
	With family	58 (34.5)	110 (65.5)	1	
Smoking	No	46 (35.1)	85 (64.9)	1	0.46
	Yes	14 (29.2)	34 (70.8)	1.3 (0.6-2.7)	
Alcohol drinking	No	42 (35.3)	77 (64.7)	1	0.48
	Yes	18 (30.0)	42 (70.0)	1.3 (0.7-2.5)	
Systemic disease	No	6 (23.1)	20 (76.9)	1.8 (0.7-4.8)	0.23
	Yes	54 (35.3)	99 (64.7)	1	
Diabetes mellitus	No	41 (33.1)	83 (66.9)	1.1 (0.6-2.1)	0.85
	Yes	19 (34.5)	36 (65.5)	1	
DMFT	≤17	34 (39.1)	53 (60.9)	1	0.13
	>17	26 (28.3)	66 (71.7)	1.6 (0.9-3.0)	
History of dental visit in the past year	No	43 (24.0)	84 (76.0)	1.6 (0.8-3.1)	0.21
	Yes	23 (12.8)	29 (87.2)	1	
Ever received dental treatment	No	9 (5.1)	23 (94.9)	2.1 (0.8-5.5)	0.14
	Yes	57 (31.8)	90 (68.2)	1	
Stress	No	47 (41.2)	67 (58.8)	1	0.005
	Yes	13 (20.0)	52 (80.0)	**2.8 (1.4-5.7)**	
Toothbrushing	Less than twice daily	26 (30.6)	59 (69.4)	1.3 (0.7-2.4)	0.43
	At least twice daily	34 (36.2)	60 (63.8)	1	
Fluoride toothpaste use	No	8 (21.1)	30 (78.9)	2.2 (0.9-5.1)	0.07
	Yes	52 (36.9)	89 (63.2)	1	

Boldface indicates statistical significance.

Table 5 presents multivariate logistic regression analyses of the associations between stress and moderate to severe periodontitis. Elderly persons with stress were 3 times more likely to have moderate to severe periodontitis compared to those without stress (adjusted OR = 3.0, 95 % CI = 1.3-7.0). The analysis was adjusted for gender, age, working status, diabetes, smoking and history of dental treatment. Similar results in model II were obtained when using Hilgert *et al.*'s definition of periodontitis. Stress was associated with an adjusted OR of 3.9 (95 % CI = 1.7-9.0) for the development of periodontitis.

Table 5 *Associations between stress and periodontal disease among elderly in Khon Kaen province in multivariate logistic regression*

Variable	Odds ratio (95 % Confidence Interval)	
	Model I	Model II
Stress	**3.0 (1.3-7.0)**	**3.9 (1.7-9.0)**
Male gender	**2.2 (1.1-4.5)**	**3.6 (1.7-7.5)**
Aged 70 years or above	1.3 (0.6-2.8)	1.1 (0.5-2.3)
Employed/actively working	**2.4 (1.2-5.0)**	**3.4 (1.5-7.2)**
Diabetes	1.3 (0.6-2.7)	2.1 (0.9-4.5)
Smoking	0.9 (0.4-2.2)	1.9 (0.8-4.8)
Ever received dental treatment	2.4 (0.9-6.5)	1.5 (0.6-3.7)

Boldface indicates statistical significance.

Discussion

Studies relating psychosocial stress to periodontal disease have been conducted for many years.[15-17] To our knowledge, this was the first epidemiologic study investigating the association between stress and periodontitis among Thai elderly. Our results support the hypothesis that stress may be associated with an increased risk of periodontitis. The present study found that persons subjected to stress have higher risk of severe attachment loss than those without stress in which potential confounders such as gender, age, occupation, diabetes mellitus, smoking, and history of dental treatment could be controlled.

The major strength of this study is that we determine the main outcome variable by measuring the CAL for evaluating periodontitis. Periodontitis is a disease that can newly develop, regress or progress over time. As an attachment loss requires long periods to develop,[4] CAL measurement can support the validity of periodontitis case ascertainment. According to the high prevalence of periodontal disease among Thai elderly[3] therefore, we categorized the subjects based on the percentage of sites with CAL≥5 mm to determine the severity of the periodontitis. Full-mouth periodontal examination (FMPE) is time-consuming in periodontal surveys then the random half-mouth six-sites per tooth protocol was used. This method produces the smallest bias and provides the best agreement with FMPE in estimating periodontitis severity as determined by CAL, PD, and BOP.[13]

The conclusion of our study is based on self-reported psychosocial traits, the reliability of the psychosocial instruments used was therefore important. "Self Analyzed and Self Evaluated Stress Test (SSST),[12] which is a standardized questionnaire in Thailand was used to measure stress level. It was developed by the Department of Mental Health, Ministry of Public Health.

This is a measure with high sensitivity and has been widely used in health service system and general population of Thailand.

Since the first study[6] provided the positive evidence that psychosocial stress was associated with periodontitis, some studies further demonstrated that stress may contribute in the etiology of periodontal disease development.[18,19] Other studies revealed that individuals under high working load, bad marital status,[20] occupational dissatisfaction,[21] and high psychological strain caused by negative life events[22] indicated more periodontal destruction. Negative life events such as the loss of a spouse may cause a change of immunological system.[23] An association between psychosocial stress assessed as financial pressure and increased attachment loss as well as alveolar bone loss has been determined.[24] All those studies provide evidence that psychological factors influence the periodontal status most likely by modulating the function of the immunological system. A systematic review has confirmed that epidemiological studies of periodontal diseases are complicated by the different criteria used to define periodontal disease.[25] Moreover; the prevalence of periodontal disease among Thai elderly is high.[3] Therefore, we categorized the subjects by tertiles based on the percentage of sites with CAL≥5 mm. Subjects were classified into three periodontitis groups: no/mild, moderate and severe to assess the extent of periodontitis. This was the same method as in the study of Sim *et al.*[26] However, in order to compare our results to other similar study, we conducted additional analysis by using Hilgert *et al.*[14] 's cut-off points with 30 % of the sites with CAL≥5 mm. Similar results were found. Hilgert *et al.*[14] found that cortisol levels were positively associated with 30 % of the sites with CAL≥5 mm (OR = 6.9; 95 % CI: 1.7 to 27.1). Our results also showed similar association between periodontitis and stress (OR of 3 with tertile of percentage of site with CAL≥5 mm and OR of 3.9 with 30 % of the sites with CAL≥5 mm.) The results indicated that individuals with higher stress level are more likely to have greater risk of having periodontitis. Compared to the report by Genco *et al.*[24] indicated that, of all the daily strains investigated, only financial strain was associated significantly with greater attachment and bone loss (OR = 1.70; 95 % CI: 1.09 to 2.65 and OR = 1.68; 95 % CI: 1.20 to 2.37, respectively). These results support the hypothesis that stress is associated with an increased risk of periodontitis.

However, the impact of stress seems to be more complex. The potential role is that stress is a mediating mechanism between individual characteristics and periodontal disease. It may lead to the lower host resistance which may mediate the putative relationship between psychosocial factors and periodontal disease.[27] Stress is proposed as an important disruptive factor in the homeostatic regulation between oral pathogenic bacteria and the host's immune system. The decreased immune reactions would offer the bacteria an opportunity to proliferate and invade the periodontal tissues. As an indirect way, stress may affect health behavior of persons with high stress by changing their habits to be harmful to periodontal condition, such as negligent oral hygiene, resulting in negative effects for the function of immune system.[9] In a laboratory studies using mice, Shapira *et al.* [28,29] found that an 'emotional' stressor (isolation) and a physical stressor (cold), compared to control group, had the effect of modifying the inflammatory response following introduction of *Porphyromonas gingivalis*, through suppression of macrophages, increased secretion of nitric oxide and reduction of tumor necrosis factor-alpha. Deinzer *et al.*[30] reported a study to determine the association between academic stress and gingival inflammation, assessing changes in interleukin-1 beta, a component of the immune system thought to play a role in periodontal tissue destruction. It revealed that examination students showed significantly higher levels of interleukin-1beta at both the experimental gingivitis sites (area under the curve, exam group: 1240.64 ± 140.07; control group: 697.61 ± 111.30; p = 0.004) and the sites of good oral hygiene (exam group: 290.42 ± 63.19; control group: 143.98 ± 42.71; p = 0.04)., indicating

that stress may affect periodontal health through suppressed immune system activity, especially when oral hygiene is neglected. In an exploratory case–control study of psychosocial factors and adult periodontitis, Moss *et al.*[31] defined cases as those individuals with active periodontal disease and who also demonstrated further periodontal attachment loss at 1-year follow up. Immune system response was examined according to median splits for level of serum immunoglobulin G (IgG) antibody to three periodontal pathogens, *Bacteroides forsythus, P*. gingivalis, and Actinomycetes actinomyce-temcomitans. The results showed significantly elevated odds ratios (OR) for cases and associated with IgG P. gingivalis (OR = 4.55) and IgG *A.actinomycetemcomitans* (OR = 5.29) while IgG *B. forsythus* was significantly associated with elevated odds for being a periodontal disease case, but only among individuals who had higher scores for daily strains. This exploratory analysis has served to identify specific lines of inquiry concerning psychosocial measures as important environmental factors in adult periodontitis.

The finding reported in our study does not establish causality. Cross-sectional study would be difficult to infer the temporal association between a risk factor and an outcome. Therefore, only an association can be inferred. Our study was limited to the young old (60 to 69) group; the mean age of this study population is 67.1 ± 5.7 years. Most people in their 60s and early 70s are still fit, active, and able to take care of themselves and may consent to take part in the study different from middle-old (75–84), and oldest-old (85+).[32] These may be prone to non-response bias therefore; the results cannot be automatically applied to middle-old, and oldest-old groups.

Suggestions for the future research are to increase the number of subjects and collect more psychosocial stress factors which would allow for analysis of possible confounding factors. Further longitudinal studies are needed to examine whether stress is an independent risk factor for periodontal disease. In addition, the findings suggest that this research highlights the importance for interdisciplinary approach between dentist and mental health personnel who involve in both preventive and curative approaches. Decision makers should give some more attention; thus, elderly will gain considerable improvement in oral health and psychological health under globalization.

Conclusions

This study provides some evidence of an association between stress and periodontitis in older adults when various potential confounders, including gender, age, occupation, diabetes mellitus, smoking, and history of dental treatment were controlled. Although our results cannot prove a causal relationship, these findings might have valuable implications for the prevention of periodontitis.

Ackonwledgment

All authors express special appreciation to all participants of this study. This study was supported by grants from Faculty of Dentistry, Khon Kaen University, Thailand.

References

1. Chayavon N, Knodel J, Siriboon S. Thailand's Elderly Population: A Demographic and Social Profile Based on Official Statistical Sources. Bangkok: Chulalongkorn University; 1997.

2. George K. Social factors and illness In Binstock, R.H. and George, L.K.(eds), Aging and Modernization. New York: Appleton-CENTURY-Crofts; 1996.

3. Bureau of Dental Health. Report of the 7th Thailand National Oral Health Survey. Bangkok: Department of health ,Ministry of Public Health; 2013.

4. Socransky SS, Haffajee AD. The bacterial etiology of destructive periodontal disease: current concepts. *J*

Periodontol 1992;63:322-31.
5. Genco RJ. Current view of risk factors for periodontal diseases. ***J Periodontol*** 1996;67:1041-9.
6. Moulton R, Ewen S, Thieman W. Emotional factors in periodontal disease. ***Oral Surg Oral Med Oral Pathol*** 1952;5:833-60.
7. da Silva AM, Newman HN, Oakley DA. Psychosocial factors in inflammatory periodontal diseases. A review. ***J Clin Periodontol*** 1995;22:516-26.
8. Breivik T, Thrane PS, Murison R, Gjermo P. Emotional stress effects on immunity, gingivitis and periodontitis. ***Eur J Oral Sci*** 1996;104:327-34.
9. Genco RJ, Ho AW, Kopman J, Grossi SG, Dunford RG, Tedesco LA. Models to evaluate the role of stress in periodontal disease. ***Ann Periodontol*** 1998;3:288-302.
10. Irwin M, Patterson T, Smith TL, Caldwell C, Brown SA, Gillin JC, *et al.* Reduction of immune function in life stress and depression. ***Biol Psychiatry*** 1990;27:22-30.
11. Biondi M, Zannino LG. Psychological stress, neuroimmunomodulation, and susceptibility to infectious diseases in animals and man: a review. ***Psychother Psychosom*** 1997;66:3-26.
12. Department of Mental health in Ministry of Public health. Self-analyzed and self-Evaluated Stress Test. Nonthaburi: Agricultural Cooperative Federation of Thailand Press; 2008.
13. Chu Y, Ouyang X. Accuracy of partial-mouth examination protocols for extent and severity estimates of periodontitis: a study in a Chinese population with chronic periodontitis. ***J Periodontol*** 2015;86:406-17
14. Hilgert JB, Hugo FN, Bandeira DR, Bozzetti MC. B. Stress, cortisol, and periodontitis in a population aged 50 years and over. ***J Dent Res*** 2006;85:324-8.
15. Marcenes WS, Sheiham A. The relationship between work stress and oral health status. ***Soc Sci Med*** 1992;35:1511-20.
16. Green LW, Tryon WW, Marks B, Huryn J. Periodontal disease as a function of life events stress. ***J Human Stress*** 1986;12:32-6.
17. Freeman R, Goss S. Stress measures as predictors of periodontal disease - A preliminary communication. ***Community Dent Oral Epidemiol*** 1993;21:176-7.
18. Pindborg JJ. Gingivitis in military personnel with special reference to ulceromembranous gingivitis. ***Odontologisk Revy*** 1951;59:407–99.
19. Goldhaber P, Giddon DB. Present concepts concerning the etiology and treatment of acute necrotizing ulcerative gingivitis. ***International Dental Journal*** 1964;14:468–96.
20. Marcenes WS, Sheiham A. The relationship between work stress and oral health status. ***Soc Sci Med*** 1992;35:1511–20.
21. Linden GJ, Mullally BH, Freeman R. Stress and the progression of periodontal disease. ***J Clin Periodontol*** 1996;23:675-80.
22. Croucher R, Marcenes WS, Torres MCMB, Hughes WS, Sheiham A. The relationship between life-events and periodontitis. A case-control study. ***J Clin Periodontol*** 1997;24:39-43.
23. Stein M, Keller SE, Schleifer SJ. Stress and immuno-modulation: the role of depression and neuroendocrine function. ***J Immunol*** 1985;135:827–33.
24. Genco RJ, Ho AW, Grossi SG, Dunford SG, Tedesco LA. Relationship of stress, distress, and inadequate coping behaviors to periodontal disease. ***J Periodontol*** 1999;70:711–23.
25. Savage A, Eaton KA, Moles DR, Needleman I. A systematic review of definitions of periodontitis and methods that have been used to identify this disease. ***J Clin Periodontol*** 2009 Jun;36:458-67.
26. Sim SJ, Kim HD, Moon JY, Zavras AI, Zdanowicz J, Jang SJ, *et al.* Periodontitis and the risk for non-fatal stroke in Korean adults. ***J Periodontol*** 2008;79:1652-8.
27. da Silva AM, Newman HN, Oakley DA. Psychosocial factors in inflammatory periodontal diseases. A review. ***J Clin Periodontol*** 1995;22:516-26.
28. Shapira L, Frolov I, Halabi A, Ben-Nathan D. Experimental stress suppresses recrutiment of macrophages but enhanced their P. gingivalis LPS-stimulated secretion of nitric oxide. ***J Periodontol*** 2000;71:476-81.
29. Shapira L, Houri-Haddad Y, Frolov I, Halabi A, Ben-Nathan

D. The effect of stress on the inflammatory response to Porphyromonas gingivalis in a mouse subcutaneous chamber model. ***J Periodontol*** 1999;70:289-93.
30. Deinzer R, Forster P, Fuck L, Herforth A, Stiller-Winkler R, Idel H. Increase of crevicular interleukin 1beta under academic stress at experimental gingivitis sites and at sites of perfect oral hygiene. ***J Clin Periodontol.*** 1999;26:1-8.
31. Moss ME, Beck JD, Kaplan BH, Offenbacher S, Weintraub JA, Koch GG, *et al*. Exploratory case-control analysis of psychosocial factors and adult periodontitis. ***J Periodontol*** 1996;67:1060-9.
32. Laura E. Berk. Development Through the Lifespan: Allyn & Bacon; 2010.

Original Article

Skeletal Age Estimation in A Group of Contemporary Thai Children and Adolescents using Tanner-Whitehouse 3 (TW3) Method

Sunpatch Benjavongkulchai[1] **and Pisha Pittayapat**[1]

[1]Department of Radiology, Faculty of Dentistry, Chulalongkorn University, Bangkok

Abstract

Objectives: To evaluate the accuracy of Tanner-Whitehouse method (TW3 RUS score) on Thai subjects.

Methods: A total of 200 hand and wrist radiographs from patients who need orthodontic treatment or other treatments were collected at the Department of Radiology, Faculty of Dentistry, Chulalongkorn University. The subjects, defined as contemporary Thais, were 8-20 years old when the radiographs were taken. Age estimation was done using Tanner- Whitehouse 3, RUS score, method (TW3-RUS) by two calibrated observers. The observation was done twice with 4-week-time interval. Comparison between the chronological ages and the estimated ages by TW3-RUS method was done. Descriptive analysis was analyzed. Mean differences between the age estimated by TW3-RUS method and the chronological age were calculated. Wilcoxon signed ranks test and Spearman's correlation coefficient were used to compare the estimated age with the chronological age. Weighted kappa analysis was used to test the intra-observer and inter-observer reliability.

Results: The mean difference between the estimated age and the chronological age showed an overall overestimation of 0.15 (standard deviation (SD) = 1.63) year. Wilcoxon signed ranks test showed statistically significant difference between the TW3-RUS estimated age and the chronological age (p = 0.02). Spearman's correlation coefficient showed significant correlation between the TW3-RUS estimated age and the chronological age (rs = 0.86, $p < 0.001$). Good intra-observer reliability was found with weighted kappa of 0.813 - 0.941. Moderate to good inter-observer reliability was found with weighted kappa 0.674 - 0.946. Ulna bone showed the lowest inter-observer reliability (kappa value = 0.674).

Conclusion: Significant differences were found between the estimated age using TW3-RUS method and the chronological age of a group of contemporary Thai children and adolescents. Further studies should be conducted on the adaptation of TW3-RUS method in order to improve its accuracy on Thai population.

Keywords: Age estimation, Hand and wrist radiography, Tanner-Whitehouse, Thai

Received Date: APR 27, 2017 **Accepted Date:** JUL 17, 2017

doi: 10.14456/jdat.2017.23

Correspondence to:

Pisha Pittayapat. Department of Radiology, Faculty of Dentistry, Chulalongkorn University 34 Henri-Dunant Road, Pathumwan, Bangkok 10330 Thailand Email address: p.pittayapat@gmail.com Tel: 081-814-2656

Introduction

Age estimation is applied as an essential part in many situations, such as growth observation, human identification, immigrant registration and legal judgment. So far, pediatricians had collected data to find norms of skeletal development and introduced several age estimation methods to make a proper comparison with patients in order to evaluate their developmental status.[1-3] When an unknown body was found, information on estimated age would help screening for a person who is possibly the victim.[2,4]

In the medical aspect, skeletal and dental developments are referred as the representation of chronological age.[5] Morphological changes and developmental stages of bones are useful indicators as well as eruption and morphological development of teeth.[5] Age estimation by using hand and wrist radiography is considered as the first choice for many cases since it is uncomplicated, inexpensive and non-invasive.[5,6]

Human hand and wrist consists of 27 bones for each side of the body. The 19 bones of one hand can be counted into 5 metacarpuses, 5 proximal phalanges, 4 middle phalanges (absent in thumb finger), and 5 distal phalanges. The rest 8 bones, called carpal bones, belong to the wrist and are defined as capitate, hamate, pisiform, triquetrum, lunate, scaphoid, trapezium, and trapezoid. There is an exceptionally calcified mass found on the thumb called a sesamoid bone. Radius bone and ulnar bone are adjacent to the wrist and found on hand and wrist radiographs thus, are also used as developmental indicators.[7]

During long bone development, epiphyseal development are defined in stages: presenting, widening, capping (cover) at the end of diaphysis, and fusing with the diaphysis.[7] Unlike long bones, the morphological stages of carpal bones are not so empirical. The last change found in hand and wrist region is a complete fusion of distal epiphysis with diaphysis of radius bone at the age of 17 years in female and 19 years in male.[8] Therefore, hand and wrist cover almost 20 years of human development, from the time of newborn to the end of teenager.

Many age estimation methods using hand and wrist radiographs have been proposed.[8-10] Each method has its own pros and cons relying on which of the main concept it belongs. One of the most recently published methods and is well-known in the anthropological field is the "Tanner-Whitehouse method".[5,10-14]

Tanner-Whitehouse method refers to stages of skeletal growth focusing on regions on hand and wrist bones. Each stage of each region is represented by a number.[10,15] The numbers corresponding with the present bone stage from all regions are then summed together and compared with the sum score table correlated with the chronological age.[10,15] This method has been introduced in 3 editions called "TW1", "TW2" and "TW3". The latest edition (TW3) was published in 2001.[6,10] TW3 uses the new data which covers more varieties of ethnicity resulting in the new sum score table. TW3 is composed of 2 scoring systems which can be used separately: "radius, ulna, and selected metacarpal and phalanges (RUS) score", relying on 13 bone (Fig. 1) (Table 1) and "carpals (CAR) score", relying on 7 carpal bones.[6,10]

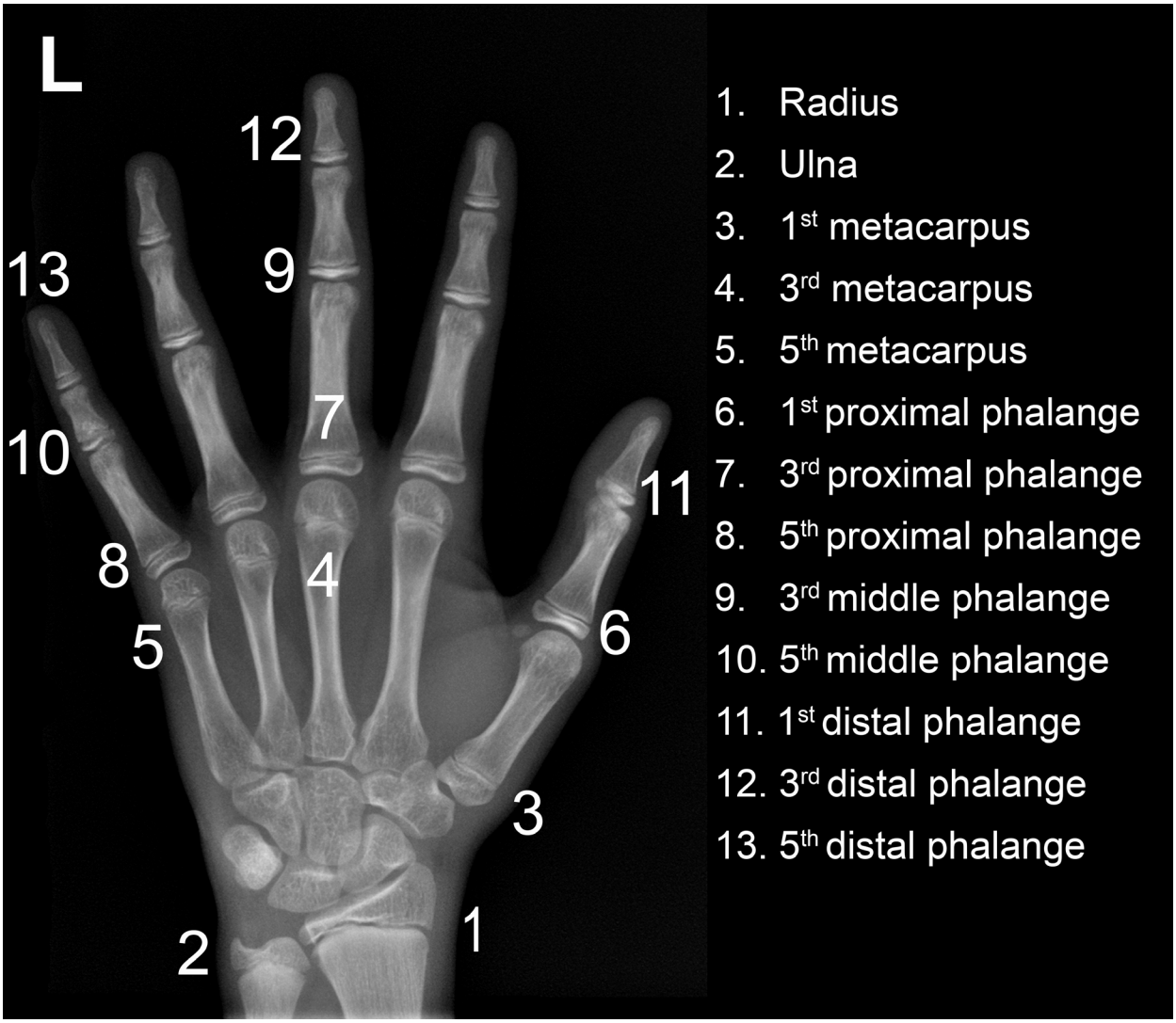

Figure 1 *An example of hand and wrist radiograph, showing 13 regions of interest of the hand and wrist bones according to Tanner and Whitehouse 3 (TW3-RUS) method. Each number referred to each bone: 1, Radius; 2, Ulna; 3, 1st metacarpus; 4, 3rd metacarpus; 5, 5th metacarpus; 6, 1st proximal phalange; 7, 3rd proximal phalange; 8, 5th proximal phalange; 9, 3rd middle phalange; 10, 5th middle phalange; 11, 1st distal phalange; 12, 3rd distal phalange; 13, 5th distal phalange. Stages based on TW3 definitions were given to each region and then translated to RUS scores (Table 1). Sums of the scores were then converted to age*

Table 1 *RUS-score according to TW3-RUS method of male (M) and female (F) for each stage of each region on a hand and wrist radiograph*

		Stage								
		A	B	C	D	E	F	G	H	I
Radius	M	0	16	21	30	39	59	87	138	213
	F	0	23	30	44	56	78	114	160	218
Ulna	M	0	27	30	32	40	58	107	181	
	F	0	30	33	37	45	74	118	173	
1st metacarpus	M	0	6	9	14	21	26	36	49	67
	F	0	8	12	18	24	31	43	53	67
3rd metacarpus	M	0	4	5	9	12	19	31	43	52
	F	0	5	8	12	16	23	37	47	53
5th metacarpus	M	0	4	6	9	14	18	29	43	52
	F	0	6	9	12	17	23	35	48	52
1st proximal phalange	M	0	7	8	11	17	26	38	52	67
	F	0	9	11	14	20	31	44	56	67
3rd proximal phalange	M	0	4	4	9	15	23	31	40	53
	F	0	5	7	12	19	27	37	44	54
5th proximal phalange	M	0	4	5	9	15	21	30	39	51
	F	0	6	7	12	18	26	35	42	51
3rd middle phalange	M	0	4	6	9	15	22	32	43	52
	F	0	6	8	12	18	27	36	45	52
5th middle phalange	M	0	6	7	9	15	23	32	42	49
	F	0	7	8	12	18	28	35	43	49
1st distal phalange	M	0	5	6	11	17	26	38	46	66
	F	0	7	9	15	22	33	48	51	68
3rd distal phalange	M	0	4	6	8	13	18	28	34	49
	F	0	7	8	11	15	22	33	37	49
5th distal phalange	M	0	5	6	9	13	18	27	34	48
	F	0	7	8	11	15	22	32	36	47

Pinchi *et al.* compared skeletal age and chronological of Italian children and adolescents using Greulich and Pyle atlas method, TW2 and TW3.[16] The results showed the median differences for TW3 and GP methods were close to 0. No significant differences were found between estimated and chronological age for TW3. TW2 proved to be the worst among the three.[16]

Some factors may influence the accuracy of the predicted age. The factors that should be taken into account are genetic variations and generation differences.[5,6]

Genetic variations affect the progress on physiological development, including skeletal development, correlating with ages.[1,6] At least 2 aspects must be taken into account: sex and ethnicity. By sex,

many previous studies found that females usually grow faster than males.[1,2,14,17-22] By ethnicity, there are many studies finding differences in timing of growth spurt and rate of skeletal growth between ethnic groups.[2,5,6,11,19,23-25]

Generation differences also affect the accuracy of age estimation. Children developmental rate tended to be faster in younger generations.[1,2,26-28] Studies on recent generation showed age overestimation using the long time-practiced age estimation methods.[5,12,26,27] Hsieh *et al.* investigated the skeletal maturation of Taiwanese children from two generations using TW3 method.[27] It was found that the skeletal maturation of children in the mid-2000s is faster than that in the mid-1960s.[27]

Since Tanner-Whitehouse 3 (TW3) method was recently revised and is more applicable with multiple ethnic groups, but no study on Thai population has been published. Therefore, the aim of this study was to evaluate the accuracy of Tanner-Whitehouse method (TW3-RUS score) on a group of contemporary Thai children and adolescents.

Materials and Methods

Samples

Hand and wrist radiographs from patients who need orthodontic treatment or other treatments were collected at the Department of Radiology, Faculty of Dentistry, Chulalongkorn University. The radiographs were taken by Carestream™ CS 8000c and CS 9000c x-ray machine (Carestream Health, Inc, Rochester, NY, USA) using standard exposure parameters based on patients' size. The subjects were 8-20 years old when the radiographs were taken. Radiographs of left or right hand and wrist were both included since no significant difference was found when using them for age estimation.[8,29,30]

Selection criteria were set in order to control the influencing factors and the subjects were defined as "contemporary Thai". The patients must have declared Thai nationality and the hand and wrist radiographs must have been taken from 1st January 2011 to 31st December 2016. Therefore, the date of birth of all subjects must be between 1991 and 2008. Patients with history of systemic diseases that affect skeletal development were excluded.

Observations

After the screening process, 200 hand and wrist radiographs were included in this study (98 males with mean age = 12.28 years, standard deviation (SD) = 2.26 years; 102 females, with mean age = 12.28 years, SD = 2.47 years) (Table 2). The included cases were randomized. Two pre-calibrated observers participated in the observation: one master student in dentomaxillofacial radiology and one dentomaxillofacial radiologist with 13 years experiences.

Table 2 *Frequency of the subjects in each age group*

Age (year)	Female	Male	Total
8 – 8.99	7	7	14
9 – 9.99	9	9	18
10 – 10.99	18	10	28
11 – 11.99	15	22	37
12 – 12.99	17	13	30
13 – 13.99	16	21	37
14 – 14.99	6	5	11
15 – 15.99	5	5	10
16 – 16.99	3	3	6
17 – 17.99	3	1	4
18 – 18.99	1	1	2
19 – 19.99	1	1	2
20 – 20.99	1	0	1
Total	102	98	200

Age estimation was done using Tanner-Whitehouse 3, RUS score, method (TW3-RUS method) (Table 1) (Fig. 1).[10] The hand and wrist images were visualized using Infinitt® PACS software (Infinitt Healthcare Co., Ltd., Seoul, South Korea). During the estimation process the observers were blinded from the true (chronological) age leaving only the sex of the patients to be known. The first observer did the age estimation on the whole samples. Twenty-percent of the samples were then randomly selected for intra- and inter-observer analysis. The first observer did the second observation on the selected 20 % of the samples 4 weeks after the first observation. The second observer performed age estimation with this group of samples for inter-observer analysis.

Statistical analysis

Comparison between the chronological ages and the estimated ages by TW3-RUS method was done. Descriptive analysis was analyzed. The samples were categorized by the chronological age, 1-year-old-ranged for each group. In each group, the mean and standard deviation of the estimated age and the chronological age were calculated. Mean differences between the age estimated by TW3-RUS method and the chronological age were also calculated. To evaluate the accuracy of the TW3-RUS age estimation method, Wilcoxon signed ranks test was used to compare the estimated age with the chronological age. The significance was set at $p<0.05$. The correlation between the estimated age and the chronological age was analyzed by Spearman's rank-order correlation. Weighted kappa analysis was used to test the intra-observer and inter-observer reliability.

Results

Tanner-Whitehouse 3, RUS score age estimation technique

The mean chronological age, mean TW3-RUS estimated age and mean age difference for each age group were shown (Table 3 - 5).

Table 3 *Mean and standard deviation (SD) of chronological age, TW3-RUS estimated age and mean differences (TW3-RUS estimated age – chronological age) for each age group regardless of sex*

Age group (year)	Mean chronological age ± SD (year)	Mean TW3-RUS estimated age ± SD (year)	Mean difference ± SD (year)
8 – 8.99	8.63 ± 0.29	8.14 ± 1.79	-0.49 ± 1.76
9 – 9.99	9.58 ± 0.22	9.62 ± 1.88	0.04 ± 1.86
10 – 10.99	10.42 ± 0.29	10.35 ± 1.53	-0.08 ± 1.57
11 – 11.99	11.45 ± 0.33	11.81 ± 1.44	0.36 ± 1.42
12 – 12.99	12.40 ± 0.30	13.15 ± 1.35	0.75 ± 1.29
13 – 13.99	13.46 ± 0.26	14.45 ± 0.38	0.73 ± 1.54
14 – 14.99	14.45 ± 0.38	14.96 ± 1.12	0.52 ± 0.97
15 – 15.99	15.54 ± 0.36	15.42 ± 0.98	-0.12 ± 0.97
16 – 16.99	16.26 ± 0.32	15.75 ± 0.82	-0.51 ± 0.66
17 – 17.99	17.39 ± 0.18	15.38 ± 0.75	-2.01 ± 0.63
18 – 18.99	18.58 ± 0.38	15.75 ± 1.06	-2.83 ± 0.69
19 – 19.99	19.64 ± 0.21	15.75 ± 1.06	-3.89 ± 0.86
20 – 20.99	20.15*	15.00*	-5.15*
overall	12.28 ± 2.37	12.43 ± 2.64	0.15 ± 1.63

**only one subject present in the study*

Table 4 *Mean and standard deviation (SD) of chronological age, TW3-RUS estimated age and mean differences (TW3-RUS estimated age – chronological age) for male subjects*

Age group (year)	Mean chronological age ± SD (year)	Mean TW3-RUS estimated age ± SD (year)	Mean difference ± SD (year)
8 – 8.99	8.63 ± 0.34	8.34 ± 1.88	-0.28 ± 1.75
9 – 9.99	9.56 ± 0.23	9.07 ± 2.25	-0.49 ± 2.27
10 – 10.99	10.51 ± 0.32	9.27 ± 1.24	-1.24 ± 1.27
11 – 11.99	11.51 ± 0.33	11.35 ± 1.64	-0.16 ± 1.54
12 – 12.99	12.37 ± 0.32	13.09 ± 1.81	0.71 ± 1.74
13 – 13.99	13.47 ± 0.27	13.94 ± 2.01	0.47 ± 1.92
14 – 14.99	14.54 ± 0.39	15.52 ± 1.08	0.98 ± 0.95
15 – 15.99	15.42 ± 0.41	15.84 ± 1.31	0.42 ± 1.14
16 – 16.99	16.45 ± 0.39	16.50 ± 0.00	0.05 ± 0.39
17 – 17.99	17.60*	16.50*	-1.10*
18 – 18.99	18.80*	16.50*	-2.30*
19 – 19.99	19.80*	16.50*	-3.30*
All males	12.28 ± 2.26	12.26 ± 2.98	-0.03 ± 1.75

**only one subject present in the study*

Table 5 *Mean and standard deviation (SD) of chronological age, TW3-RUS estimated age and mean differences (TW3-RUS estimated age – chronological age) for female subjects*

Age group (year)	Mean chronological age ± SD (year)	Mean TW3-RUS estimated age ± SD (year)	Mean difference ± SD (year)
8 – 8.99	8.63 ± 0.26	7.93 ± 1.81	-0.71 ± 1.87
9 – 9.99	9.59 ± 0.24	10.18 ± 1.32	0.58 ± 1.24
10 – 10.99	10.37 ± 0.27	10.94 ± 1.35	0.57 ± 1.35
11 – 11.99	11.35 ± 0.31	12.49 ± 0.68	1.14 ± 0.72
12 – 12.99	12.42 ± 0.28	13.20 ± 0.91	0.78 ± 0.87
13 – 13.99	13.44 ± 0.27	14.52 ± 0.71	1.08 ± 0.74
14 – 14.99	14.37 ± 0.40	14.50 ± 1.00	0.13 ± 0.89
15 – 15.99	15.65 ± 0.30	15.00 ± 0.00	-0.65 ± 0.30
16 – 16.99	16.07 ± 0.02	15.00 ± 0.00	-1.07 ± 0.02
17 – 17.99	17.32 ± 0.15	15.00 ± 0.00	-2.32 ± 0.15
18 – 18.99	18.30*	15.00*	-3.30*
19 – 19.99	19.50*	15.00*	-4.50*
20 – 20.99	20.15*	15.00*	-5.15*
All females	12.28 ± 2.47	12.60 ± 2.27	0.32 ± 1.50

**only one subject present in the study*

Comparison between the TW3-RUS estimated age and the chronological age showed overall overestimation of 0.15 year. The mean difference for female subjects was 0.32 (SD = 1.50) year and -0.03 (SD = 1.75) year for male subjects. The data was not normally distributed (from Shapiro-Wilk test), thus Wilcoxon signed ranks test was selected to analyze the difference. The results showed statistically significant difference between the TW3-RUS estimated age and the chronological age ($p = 0.02$).

Based on the different age groups (Table 3), the differences between the chronological age and the estimated age can be categorized in 3 parts. The first part was 8 - 10 years group which the TW3-RUS age showed an underestimating trend. The estimated age of this part was -0.14 (SD = 1.69) year. The second part was between 11 and 15 years that overestimation was found in the majority. The mean difference in this part was 0.60 (SD = 1.39) year. The final part, 15 -20 years old expressed an overall underestimation of 1.23 (SD = 1.65) year.

Correlations between the chronological age and the estimated age

The results from Spearman's correlation coefficient analysis showed significant correlation between the TW3-RUS estimated age and the chronological age for both male and female subjects ($p < 0.001$) (Table 6). The overall correlation coefficient (rs) was 0.86.

Table 6 *Correlation coefficient (rs) and p-value from Spearman's rank-order coefficient analysis*

sex	Correlation coefficient (rs)	*p*-value
All	0.86	< 0.001
Male	0.85	< 0.001
Female	0.91	< 0.001

Intra- and inter-observer reliability

Weighted kappa analysis showed good agreement for the intra-observer reliability and moderate to good agreement for inter-observer reliability. The agreements on staging were separately analyzed for each bone (Table 7). The result for intra-observer analysis ranged from 0.813 to 0.941 that the third distal phalange showed the lowest reliability and the fifth proximal phalange had the highest reliability. The inter-observer reliability results showed kappa values 0.674 - 0.946. The ulna showed the lowest inter-observer reliability and the first metacarpus showed the highest reliability.

Table 7 *Weighted kappa results for intra-observer and inter-observer reliability*

	Intra-observer reliability	Inter-observer reliability
Radius	0.858	0.783
Ulna	0.848	0.674
1st metacarpus	0.854	0.946
3rd metacarpus	0.852	0.707
5th metacarpus	0.879	0.807
1st proximal phalange	0.894	0.909
3rd proximal phalange	0.926	0.863
5th proximal phalange	0.941	0.896
3rd middle phalange	0.925	0.849
5th middle phalange	0.933	0.876
1st distal phalange	0.909	0.881
3rd distal phalange	0.813	0.833
5th distal phalange	0.893	0.829

Discussion

In the present study, total of 200 hand and wrist radiographs from a group of contemporary Thai children and adolescents were investigated. Age estimation by TW3-RUS method was accomplished for all samples and then compared with the chronological age.

The TW3-RUS score was claimed to be more reliable than CAR score.[10] The development of short bones are more consistent than the carpal bones and only 11 short bones with radius and ulna are enough for age estimation.[10] In addition, the morphological differentiation of carpal bones reaches their limits earlier than of the radius and ulna, making narrower range of age prediction in CAR score. From these reasons, TW3-RUS score was chosen in this study.

The results showed a statistically significant difference between the TW3-RUS estimated age and the chronological age (p = 0.02) with average mean age difference of -0.03 (SD = 1.75) year for males and 0.32 (SD = 1.50) year for females. This was possibly due to the effect of ethnicity on skeletal maturation. Nutritional factors and socio-economic condition of people in different countries might also play a role. TW3-RUS method was studied based on European and American population whose ethnicity was Caucasian.[10] The ethnicity of Thais is mostly Southeast Asian. A few studies on Mongoloid populations were done using TW3 methods.[31] Kim *et al.* published a study on Korean children. The researchers compared the reliability of the Greulich and Pyle method, TW3 method and Korean standard bone age chart.[32] Significant correlations were found between chronological age and bone age estimated by all three methods. However, the study used samples whose age ranged between 7-12 years old and were all Mongoloid from Korean. Differences of the characteristics of the samples could explain the reason why the results were not corresponding to the result of the present study.[32] An Asian study in China found a significant different between the chronological age and TW3 estimated age.[33] Zhang *et al.* evaluated bone age of Han Chinese children aged 1-20 years. It was found that the skeletal maturity of the Chinese boys and girls differed significantly from that of TW3 after 6 years for boys and 10 years for girls.[33] Their results were corresponding to ours although the present study could not include children whose age younger than 8 years old. Han Chinese is one of the Chinese ethnicities that distributed in the Southeast Asian countries thus might explain the similar trend of results.

However, a few studies showed no significant differences.[16,34] Pinchi *et al.* found no significant differences between the estimated age and chronological age for TW3.[16] Haiter-Neto *et al*. evaluated three age estimation methods: Greulich and Pyle, TW3 and Eklöf and Ringertz.[34] Results showed no significant difference between the chronological age and the estimated age using the three methods.[34] The main factor that led the results to another direction might be from the differences in ethnicities of the samples (Italian, Brazilian) and their socio-economic condition and nutritional factors.

Another factor which may contribute to the discrepancy in the results is the generation difference. The present study refers 'contemporary' as the people born between 1991 and 2008. TW3 method was proposed in 2001.[10] The reference samples in the method must have born in 1960s – 1990s. The effect of secular change cannot be left out as there is a huge difference in nutritional shift just within one decade. A study on the effect of secular change on skeletal maturation was done by Hsieh *et al.* on Taiwanese children using TW3 method.[27] The authors concluded that the skeletal maturation of children in the mid-2000s was faster than that in the mid-1960s. The authors also suggested that the causes of the differences might be the difference in socio-economic status and difference in food consumption between the two generations.[27]

The present research is a retrospective study. The hand and wrist radiographs in this study were primarily taken for an evaluation of skeletal growth prior to orthodontic treatment. On some radiographs the position of the hands was not strictly adjusted; therefore, some bones were not aligned totally parallel to the image receptor, making it difficult to visualize the stages of bone development (e.g. capping, partial fusion of epiphysis and diaphysis) due to the overlapping and superimposition of bones. The thumb finger was the most problematic part as the finger torsion was different from which was illustrated in the original TW3 method.[10] However, the overall quality of the radiographs was acceptable.

Observer dependence is another factor that might influence the reliability of the estimated age.[6,35-38] In this study, kappa analysis showed good intra-observer reliability and moderate to good inter-observer reliability (Table 7). The ulna bone showed lowest reliability for both intra- and inter-observer agreement. Experiences of the observers on hand and wrist radiography also played a role despite a calibration session performed prior to the observation. The definition of radiographic findings between different stages might be unclear and can still be improved.

The Spearman's analysis proved the presence of correlation between the estimated age and the chronological age, showing that TW3-RUS method was still applicable for contemporary Thais. However, since Wilcoxon signed ranks test showed that the result from the TW3-RUS age estimation was significantly different from the chronological age, some adaptation should be applied to the estimation process in case of Thai subjects. Firstly, a suggestion was made to adapt TW3-RUS for Thai population by adding the mean difference to the original result based on the age group which may increase or decrease the final predicted age. However, care must be taken when adding the mean difference to the predicted age. A validation of this adaptation still needs to be proven with more scientific evidence on another group of Thai population.

Secondly, this study showed that the skeletal age tended to be underestimated in subjects over 17 years because the maximum predicted ages of the TW3 method are 15 years old in girls and 16.5 years old in boys. Therefore, if every bone reaches the highest stage, especially if radius and ulna showed complete fusion, the result is rather unreliable and may only be concluded that the predicted age is a minimal estimated age for the individual.

Although the estimated age in this study was statistically significant different from the chronological age, TW3-RUS method showed a potential to be used on Thai children and adolescents. Further studies should still be done in order to adapt and possibly simplify TW3 method to be more applicable for Thais. In addition, the carpal bones in TW3-CAR method as claimed to be more consistent between ethnicities should also be further studied and to compare with results from TW3-RUS scoring method.

Conclusion

Significant differences were found between the estimated age using TW3-RUS method and the chronological age of a group of contemporary Thai children and adolescents. Further studies should be conducted on the adaptation of TW3 method in order to improve its accuracy on Thai population.

Acknowledgement

The Scholarship from the Graduate School, Chulalongkorn University to commemorate the 72nd anniversary of his Majesty King Bhumibala Aduladeja is gratefully acknowledged.

References

1. Berk LE. Child development. 9 ed. London, UK: Pearson Education; 2012.
2. Black S, Ferguson E. Forensic anthropology: 2000 to 2010. Boca Raton, FL: CRC Press; 2016.
3. Kliegman RM, Stanton BF, St Geme JW, Schor NF. Nelson textbook of pediatrics. 20 ed. Philadelphia, PA: Elsevier; 2015.
4. Sharma RK. Concise textbook of forensic medicine & toxicology. New Delhi, India: Elsevier India Pvt. Limited; 2007.
5. Smith T, Brownlees L. Age assessment practices: a literature review & annotated bibliography. New York, NY: Child Protection Section, United Nations Children's Fund; 2011. p. 1-78.
6. Hackman L. Age estimation in the living: a test of 6 radiographic methods: University of Dundee; 2012.
7. Schoenwolf GC, Bleyl SB, Brauer PR, Francis-West PH. Larsen's human embryology. Philadelphia, PA: Elsevier Health Sciences; 2014.
8. Greulich WW, Pyle SI. Radiographic atlas of skeletal development of the hand and wrist. Stanford, CA: Stanford University Press; 1959.
9. Fishman LS. Maturational patterns and prediction during adolescence. ***Angle Orthod*** 1987;57:178-93.
10. Tanner J, Healy M, Goldstein H, Cameron N. Assessment of skeletal maturity and prediction of adult height (TW3). London, UK: WB Saunders; 2001.
11. Buken B, Safak AA, Buken E, Yazici B, Erkol Z, Erzengin ÖU. Is the Tanner–Whitehouse (TW3) method sufficiently reliable for forensic age determination of Turkish children? ***Turk J Med Sci*** 2010;40:797-805.
12. Franklin D, Flavel A, Noble J, Swift L, Karkhanis S. Forensic age estimation in living individuals: methodological considerations in the context of medico-legal practice. ***Res Rep Forensic Med Sci*** 2015;5:53-66.
13. Satoh M. Bone age: assessment methods and clinical applications. ***Clin Pediatr Endocrinol*** 2015;24:143-52.
14. Molinari L, Gasser T, Largo RH. TW3 bone age: RUS/CB and gender differences of percentiles for score and score increments. ***Ann Hum Biol*** 2004;31:421-35.
15. Tanner JM, Whitehouse R, Marshall W, Healty M, Goldstein H. Assessment of skeletal maturity and prediction of adult height (TW2 method). Cambridge, MA: Academic Press; 1975.
16. Pinchi V, De Luca F, Ricciardi F, Focardi M, Piredda V, Mazzeo E, *et al.* Skeletal age estimation for forensic purposes: A comparison of GP, TW2 and TW3 methods on an Italian sample. ***Forensic Sci Int*** 2014;238:83-90.
17. Beunen GP, Rogol AD, Malina RM. Indicators of biological maturation and secular changes in biological maturation. ***Food Nutr Bull*** 2006;27:S244-56.
18. Bogin B, MacVean RB. The relationship of socioeconomic status and sex to body size, skeletal maturation, and cognitive status of Guatemala City school children. ***Child Dev*** 1983;54:115-28.
19. Cole TJ, Rousham EK, Hawley NL, Cameron N, Norris SA, Pettifor JM. Ethnic and sex differences in skeletal maturation among the Birth to Twenty cohort in South Africa. ***Arch Dis Child*** 2015;100:138-43.
20. Fitzpatrick LA. Sex differences in skeletal development. Advances in Molecular and Cell Biology. Volume 34. Amsterdam, Netherland: Elsevier; 2004. p. 229-45.
21. Flory CD. Sex differences in skeletal development. ***Child Dev*** 1935;6:205-12.
22. Flory CD. Osseous Development in the Hand as an Index of Skeletal Development. ***Monogr Soc Res Child Dev*** 1936;1:i+iii-ix+1-141.
23. Buken B, Safak AA, Yazici B, Buken E, Mayda AS. Is the assessment of bone age by the Greulich-Pyle method reliable at forensic age estimation for Turkish children? ***Forensic Sci Int*** 2007;173:146-53.
24. Manzoor Mughal A, Hassan N, Ahmed A. Bone age assessment methods: a critical review. ***Pak J Med Sci*** 2014;30:211-5.
25. Zhang A, Sayre JW, Vachon L, Liu BJ, Huang HK. Racial differences in growth patterns of children assessed

on the basis of bone age. ***Radiology*** 2009;250:228-35.
26. Hawley NL, Rousham EK, Norris SA, Pettifor JM, Cameron N. Secular trends in skeletal maturity in South Africa: 1962-2001. ***Ann Hum Biol*** 2009;36:584-94.
27. Hsieh CW, Liu TC, Jong TL, Tiu CM. Long-term secular trend of skeletal maturation of Taiwanese children between agricultural (1960s) and contemporary (after 2000s) generations using the Tanner-Whitehouse 3 (TW3) method. ***J Pediatr Endocr Met*** 2013;26:231-7.
28. Lai EHH, Chang JZC, Yao CCJ, Tsai SJ, Liu JP, Chen YJ, *et al.* Relationship between age at menarche and skeletal maturation stages in Taiwanese female orthodontic patients. ***J Formos Med Assoc*** 2008;107:527-32.
29. Baer MJ, Djrkatz J. Bilateral asymmetry in skeletal maturation of the hand and wrist: a roentgenographic analysis. ***Am J Phys Anthropol*** 1957;15:181-96.
30. Hackman L, Black S. Does mirror imaging a radiograph affect reliability of age assessment using the Greulich and Pyle atlas? ***J Forensic Sci*** 2012;57:1276-80.
31. Serinelli S, Panetta V, Pasqualetti P, Marchetti D. Accuracy of three age determination X-ray methods on the left hand-wrist: a systematic review and meta-analysis. ***Leg Med (Tokyo)*** 2011;13:120-33.
32. Kim JR, Lee YS, Yu J. Assessment of bone age in prepubertal healthy Korean children: comparison among the Korean standard bone age chart, Greulich-Pyle method, and Tanner-Whitehouse method. ***Korean J Radiol*** 2015;16:201-5.
33. Zhang SY, Liu LJ, Wu ZL, Liu G, Ma ZG, Shen XZ, *et al.* Standards of TW3 skeletal maturity for Chinese children. ***Ann Hum Biol*** 2008;35:349-54.
34. Haiter-Neto F, Kurita LM, Menezes AV, Casanova MS. Skeletal age assessment: a comparison of 3 methods. ***Am J Orthod Dentofacial Orthop*** 2006;130:435 e15-20.
35. Alcina M, Lucea A, Salicrú M, Turbón D. Reliability of the Greulich & Pyle method for bone age estimation in a Spanish sample. ***J Forensic Leg Investig Sci*** 2015;1:1-6.
36. King DG, Steventon DM, O'Sullivan MP, Cook AM, Hornsby VP, Jefferson IG, *et al.* Reproducibility of bone ages when performed by radiology registrars: an audit of Tanner and Whitehouse II versus Greulich and Pyle methods. ***Br J Radiol*** 1994;67:848-51.
37. Kim SY, Oh YJ, Shin JY, Rhie YJ, Lee KH. Comparison of the Greulich-Pyle and Tanner Whitehouse (TW3) methods in bone age assessment. ***J Korean Soc Pediatr Endocrinol*** 2008;13:50-5.
38. Paxton ML, Lamont AC, Stillwell AP. The reliability of the Greulich–Pyle method in bone age determination among Australian children. ***J Med Imaging Radiat Oncol*** 2013;57:21-4.

Journal of the Dental Association of Thailand
Dental Faculty Consortium of Thailand

Supported for publication from Dental Association of Thailand