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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 17th International Scientific Conference of the Dental Faculty Consortium of Thailand (DFCT2019).

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 36 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 17th International Scientific Conference of the Dental Faculty Consortium of Thailand 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, Khon Kaen 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 Khon Kaen University.

Dr. Chavalit Karnjanaopaswong
President of The Dental Association of Thailand

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Original Article

Improved Knowledge about HPV in Thai Women after Educational Intervention

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Abstract

The aim of this study was to evaluate whether the awareness and knowledge about human papillomavirus (HPV) and its association with human diseases could be improved after an educational intervention was given in 2 groups of Thai women. The participants included 370 and 348 participants in the ≤25-year-old group and the >25-year-old group, respectively. After the questionnaire composed of 15 knowledge and 2 awareness questions had been given to each participant, a knowledge brochure was distributed, then the knowledge was reassessed. Paired-T test was used to evaluate the knowledge before and after the intervention. Mean knowledge scores were increased in the ≤25-year-old (4.35±4.39 to 12.32±3.1) and the >25-year-old (4.82±4.33 to 13.22±2.35) groups. Statistically significant difference was found in both groups. The >25-year-old group had significantly higher mean knowledge score after the intervention than that of the ≤25-year-old group (13.22±2.35 VS 12.32±3.1). Most of the participants had the improved knowledge about the characteristics and pathogenesisof HPV. If proper educational intervention was given, the awareness and knowledge about HPV and HPV vaccines could be improved among female Thai participants. Therefore, giving knowledge about HPV should be considered as a public health strategy in health promotion for all Thai women.

Keywords: Educational intervention, Human papilloma virus, Knowledge, Thai

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Introduction

HPV infection is considered to be a sexually transmitted disease. It can be spread from one person to another person through anal, vaginal and oral sex or through skin-to-skin contact during sexual activity.[1,2] It has been found that during 2013–2014, the prevalence of any and high-risk genital HPV in women aged 18–69

years was 39.9 % and 20.4 %, respectively.[3] Low-risk HPV infection can lead to wart-like lesions of the skin, anogenital region and oral mucosa. High-risk HPV infection can lead to cervical cancer. HPVs also contribute to the emergence of oral benign and malignant lesions.[4] Oral potentially malignant disorders (OPMD) including oral leukoplakia, erythroplakia and erythroleukoplakia are also associated with the presence of HPV.[5] Studies have reported the prevalence rates of HPV-associated OPMD to range from 0 % to 85 %. HPVs 16 and 18 have been identified in leukoplakia.[5]

Knowledge and awareness of human papillomavirus and its association with benign and malignant lesions was investigated in several groups of population especially in Asia. A previous study in Thailand by Charakorn *et al.* demonstrated that 40.1 % of Thai women aged 17-72 years old never heard about HPV and only 38.5 % heard about HPV vaccines.[6] Since there were few reports regarding knowledge and awareness of HPV and its association with human diseases among women aged 12 and above in Thailand, therefore, the objective of this study was to evaluate the knowledge and awareness of HPV and its association with human diseases in 2 groups of Thai women including the women aged equal to or younger than 25 years old (≤25-year-old group) and the women older than 25 years old (>25-year-old group).

Materials and Methods

Ethical consideration

The study protocol was approved by the Faculty of Dentistry/Faculty of Pharmacy Mahidol University Institutional Review Board (MU-DT/PY-IRB2016-DT028).

Study populations

This study recruited convenience samples to participate in this survey. All participants were Bangkok residents. These participants included students in a high school, patients who sought treatment at the Faculty of Dentistry, Mahidol University and some participants from some public locations such as the Bangkok Mass Transit System (BTS). The study subjects were divided into 2 groups according to the ages of the participants in the educational and working fields, 370 women in the <25-year-old group (aged between 12-25 years old) and 348 women in the >25-year-old group (aged 26 to 70 years old). Inclusion criteria were participants who were Thai women who lived in Bangkok and could read and write Thai (if the participants could not read, but they wanted to take part in this study, the researcher read all the questions and wrote all the answers for them). Exclusion criteria were participants who had a problem in communication or disagreed to participate in this study.

Data collection

The questions were separated into 3 sections including; part 1 participant's demographic data; part 2 awareness questions about HPV and HPV vaccines and how they can get the information regarding knowledge about HPV and; part 3 fifteen knowledge questions about HPV infection, HPV and its association with human diseases and HPV vaccines (Table 1). The participants options to answer were yes, no or don't know. The content validity of the test was evaluated by three selected experts. All questions were agreed upon by the experts with the Index of Item Objective Congruence (IOC) ≥0.5.[7] Cronbach's alpha of 0.824 assessed the internal consistency reliability of the test. Study design was represented in the following diagram (Fig. 1).

Data analysis

Data were analyzed using the Statistical Package for the Social Sciences (SPSS) for Window Version 18.0 (SPSS Inc., Chicago, IL, USA; licensed for Mahidol University) with statistical significance of $p<0.05$. Numbers, percentage, mean and SD were used to describe participants' characteristics and awareness of HPV and HPV vaccines. Percentage was used to describe proportion of participants who answered correctly in each knowledge question. *T*-test was used to compare the mean knowledge scores between the 2 age groups and paired *t*-test was used to compare the mean knowledge scores before and after educational intervention in each group. Chi-square test was used to determine the differences in the variables between participants.

Table 1 *Fifteen knowledge questions used in the study.*

Statement (correct answer)
1. HPV can be transmitted through oral, vaginal and anal sexual conduct. (Yes)
2. Men will not have any opportunities to be infected with HPV. (No)
3. Women can be infected with HPV if they have sex when they were young. (Yes)
4. Having multiple partners increases the risk of getting HPV. (Yes)
5. Use of condoms during sexual intercourse can reduce the risk of HPV infection. (Yes)
6. Persons who are infected cannot eradicate HPV and will finally have cancer. (No)
7. HPV can lead to genital warts. (Yes)
8. HPV is the virus that can cause cervical cancer. (Yes)
9. HPV can cause oral cancers. (Yes)
10. HPV can cause other oral lesions which are not oral cancers. (Yes)
11. The HPV vaccine can prevent only some types of HPVs. (Yes)
12. HPV vaccines will be effective after the first sexual intercourse. (No)
13. HPV vaccines are effective in any age groups. (No)
14. HPV vaccines can eradicate HPV in persons who are already infected. (No)
15. Injection of HPV vaccine only once will be sufficient for the protection of HPV infection. (No)

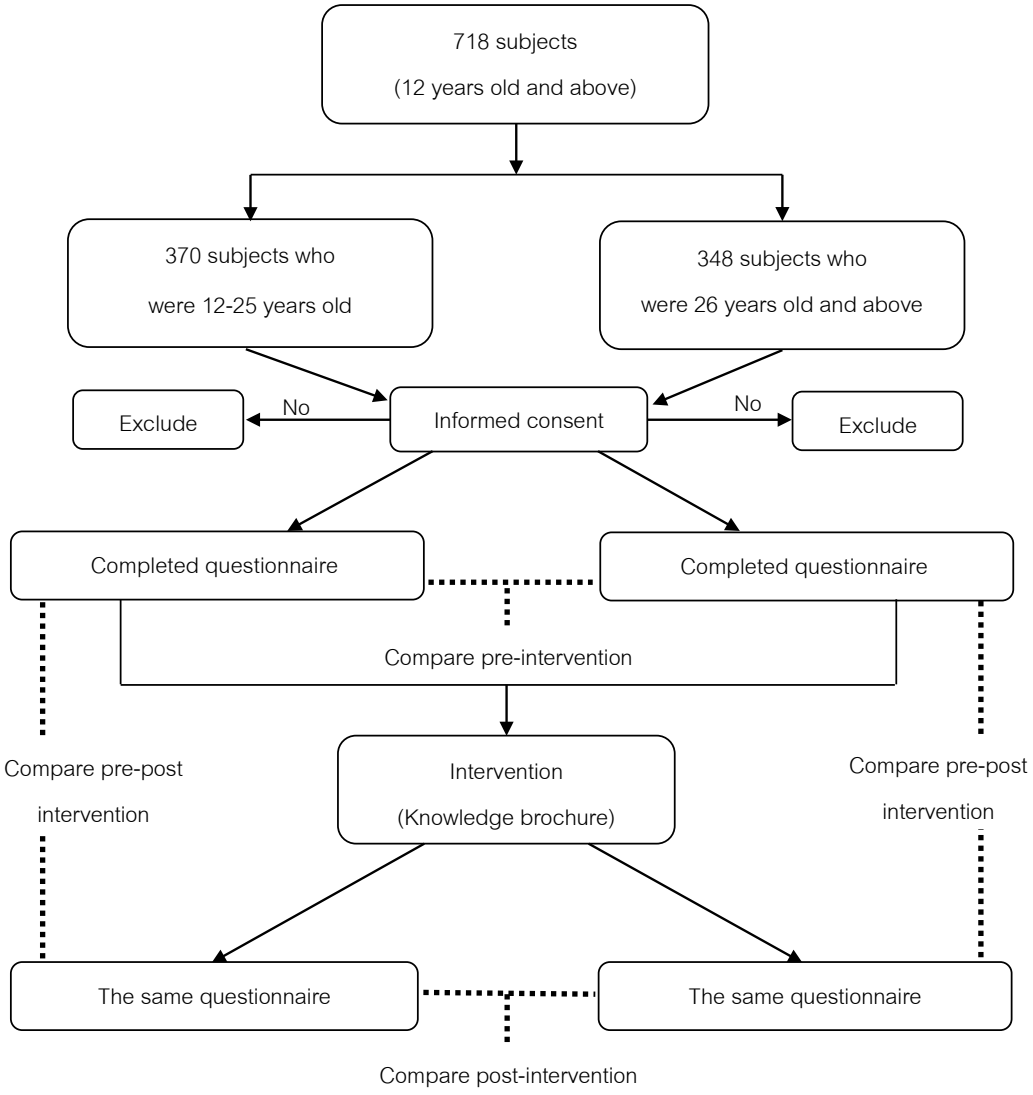

Figure 1 *Study design diagram*

Results

Participant characteristics

The demographic data of the participants were shown in Table 2. The mean age in the ≤25-year-old group was 18.1 years old (SD=3.38). Most participants in the ≤25-year-old group were high school students (66.76 %) and single (99.19 %). On the other hand, the mean ages in the >25-year-old group was 40.5 years old (SD=11.33). Most of them graduated from universities with Bachelor's degree (58.62 %) and were single (56.32 %). Regarding history of sexual debut, 36 (9.73 %) and 204 (58.62 %) of the participants from the ≤25-year-old and >25-year-old reported that they had had sex before (Table 2). Most of the participants in both groups never received HPV vaccine and most of them in the ≤25- year-old group never undergone a pap smear. Approximately half of the participants in the >25-year-old group used to undergo pap-smear in the past.

Table 2 *Demographic data of the participants.*

Characteristics	≤25-year-old group (n=370)		>25-year-old group (n=348)	
	Number	(%)	Number	(%)
Age (Years)				
12-20	280	(75.68)		
21-25	90	(24.32)		
26-30			83	(23.85)
31-40			121	(34.77)
41-50			61	(17.53)
51-60			58	(16.67)
61-70			25	(7.18)
Education attainment				
Primary school	1	(0.27)	4	(1.15)
Secondary school	247	(66.76)	12	(3.45)
Diploma	9	(2.43)	24	(6.90)
Bachelor's degree	103	(27.84)	204	(58.62)
Higher than Bachelor's degree	10	(2.70)	104	(29.88)
Marital status				
Single	367	(99.19)	196	(56.32)
Married	2	(0.54)	127	(36.50)
Divorced/Separated/Widowed	1	(0.27)	25	(7.18)
Have you ever:				
Had sexual debut				
Yes	36	(9.73)	204	(58.62)
No	319	(86.22)	80	(22.99)
Did not answer	15	(4.05)	64	(18.39)
Received HPV vaccines				
Yes	71	(19.19)	44	(12.64)
No	243	(65.68)	291	(83.62)
Not sure	56	(15.13)	13	(3.74)
Undergone pap smear testing				
Yes	8	(2.16)	186	(53.45)
No	307	(82.97)	137	(39.37)
Not sure	55	(14.87)	25	(7.18)

Awareness about HPV and HPV vaccines

Awareness about HPV and HPV vaccines was acquired by assessing participants' responses to the questionnaire. Table 3 demonstrated the number and percentage of participants who heard about HPV and HPV vaccines. Approximately 16 % from the ≤25-year-old group and 40 % from the >25-year-old group heard about HPV. Approximately 29 % from the ≤25-year-old group and 54 % from the >25-year-old group heard about HPV vaccines. There was significant difference of both awareness about HPV and HPV vaccines between the 2 age groups (P<0.001). More than half of the participants in the ≤25-year-old group never heard about HPV and HPV vaccines whereas less than half of the >25-year-old group never heard about them.

Table 3 *Awareness about HPV and HPV vaccines.*

Statement	Total (N=718)			≤25-year-old (1) group (n=370)			≤25-year-old (1) group (n=370)			Chi-Square (1)-(2)
	Yes (%)	No (%)	Don't Know (%)	Yes (%)	No (%)	Don't Know (%)	Yes (%)	No (%)	Don't Know (%)	
1. Have you ever heard of the human papillomavirus (HPV) before?	198 (27.6)	383 (53.3)	137 (19.1)	59 (15.9)	241 (65.1)	70 (19)	139 (39.9)	142 (40.8)	67 (19.3)	< 0.001*
2. Have you ever heard of the HPV vaccines before?	295 (41.1)	320 (44.6)	103 (14.3)	107 (28.9)	203 (54.9)	60 (16.2)	188 (54)	117 (33.6)	43 (12.4)	< 0.001*

** Significant difference*

Knowledge about HPV

Knowledge about HPV, HPV infection and its association with human diseases were assessed in 718 participants. Table 4 demonstrated that participants in both the ≤25-year-old and the >25-year-old groups had very little knowledge about HPV and HPV vaccine before the intervention. Similar mean knowledge scores in the ≤25-year-old (4.35±4.39) and the >25-year-old (4.82±4.33) groups were observed. After educational intervention was given, the mean knowledge scores were increased significantly in both groups (p<0.001). In addition, the >25- year-old group had significantly higher mean knowledge score compared to that of the ≤25-year-old group. Most of the participants did not know whether HPV can cause oral cancer or not. Moreover, they did not know whether HPV can cause other oral lesions which are not oral cancers (Table 5).

Table 4 *Mean knowledge scores in the ≤25-year-old and the >25-year-old groups before and after the intervention.*

	Total (N=718) Mean±SD	≤25-year-old group (n=370) Mean±SD	>25-year-old group (n=348) Mean±SD	*p*-values (≤25-year-old VS >25-year-old groups)
Before	4.58±4.36	4.35±4.39	4.82±4.33	0.148
After	12.76±2.80	12.32±3.1	13.22±2.35	<0.001*
p-values (before vs after intervention)	<0.001*	<0.001*	<0.001*	

** Significant difference*

Table 5 *Participants who answered correct answers regarding the role of HPV in causing oral lesions.*

Statement (correct answer)	Total (N=718)			≤25-year-old group (n=370)			>25-year-old group (n=348)		
	Yes (%)	No (%)	Don't Know (%)	Yes (%)	No (%)	Don't Know (%)	Yes (%)	No (%)	Don't Know (%)
9. HPV can cause oral cancers. (Yes)									
Before intervention	151 (21.03)	33 (4.60)	534 (74.37)	91 (24.60)	11 (2.97)	268 (72.43)	60 (17.24)	22 (6.32)	266 (76.44)
After intervention	654 (91.09)	26 (3.62)	38 (5.29)	327 (88.38)	16 (4.32)	27 (7.30)	327 (93.97)	10 (2.87)	11 (3.16)
10. HPV can cause other oral lesions which are not oral cancers. (Yes)									
Before intervention	100 (13.93)	38 (5.29)	580 (80.78)	51 (13.78)	27 (7.30)	292 (78.92)	49 (14.08)	11 (3.16)	288 (82.76)
After intervention	571 (79.53)	61 (8.49)	86 (11.98)	267 (72.16)	43 (11.6)	60 (16.22)	304 (87.36)	18 (5.17)	26 (7.47)

Discussion

We conducted this study to investigate the knowledge and awareness of HPV, HPV infection and HPV vaccines in a group of Thai women residing in Bangkok. We separated these participants into 2 groups since we would like to know whether teenagers and adolescents in our study knew about HPV and HPV vaccine. In addition, we added an intervention by having our participants read the brochure about HPV and its association with human diseases and the prevention of HPV infection then asked the participants to answer these questions again. Hence, we knew that the poor knowledge and awareness were from the lack of information and not from the participants' ignorance.

When the awareness of HPV and HPV vaccines was investigated, the awareness was low in the ≤25-year-old group, but was moderate in the >25-year-old group (Table 3). Despite the high prevalence of HPV, numerous studies have shown consistently that awareness of this disease is limited. Overall, 27.6 % and 41.1 % of the women in our study heard about HPV and HPV vaccines respectively (Table 3). Young participants tended to know less about HPV and HPV vaccines with 15.9 % and 28.9 %, respectively compared to older participants with 39.9 % and 54 % knowing about HPV and HPV vaccines. This result was the same as many studies in Europe and Asia. A systematic review in Europe reported that the awareness of HPV among adolescents aged 13-20 years was moderately low ranging from 5.4-66 % and the awareness of HPV vaccine was very low as well.[8]

Knowledge about HPV, HPV infection and its association with human diseases was assessed in 718 participants. Table 4 demonstrated that participants in both the ≤25-year-old and the >25-year-old groups had very little knowledge about HPV and HPV vaccine before the intervention. Similar mean knowledge scores in the ≤25-year-old (4.35±4.39) and the >25-year-old (4.82±4.33) groups were observed. After the intervention by giving a brochure of HPV and HPV vaccine information, the >25-year-old group had significantly higher mean knowledge score than that of the ≤25-year-old group (13.22±2.35 VS 12.32±3.1). In addition, statistically significant difference was found between before and after the intervention in both groups. Most of the participants had improved knowledge about the characteristics of

HPV and how the HPV infected human. Interestingly, when the questions regarding the knowledge about the role of HPV in causing oral diseases were considered, both participants from the ≤25-year-old and the >25-year-old groups did not know that HPV can cause oral diseases including oral cancers (Table 5). Nevertheless, after educational intervention, the knowledge was improved. Therefore, dentists, as one of the health care professionals, should give knowledge to their patients regarding the role of HPV in causing oral diseases.

The awareness of HPV in our participants (27.6 %) were lower compared to the previous studies conducted in Thailand. The study in 2011 by Charakorn *et al.* reported that approximately 40 % of Thai women attending the gynecology clinic aged 17-72 years old had heard about HPV.[6] However, The HPV vaccine awareness was similar in our study (41.1 %) compared to the study conducted by Charakorn (40 %). In another study in 2012, Juntasopeepun *et al.* conducted a study regarding the knowledge of HPV in the university students aged 18-25 years old. Fifteen knowledge questions were used to evaluate the knowledge of HPV and its association with human diseases and the number of questions was the same as in our study. The mean knowledge score was 7.5±3.8 out of 15.[9] In comparison to the participants in our ≤25-year- old group, the score was only 4.35±4.39 out of 15[9] which was lower than that study.

Conclusion

Awareness of HPV is poor in both the ≤25-year-old and the >25-year-old groups. Awareness of HPV vaccine is higher in the >25-year-old group compared to that of the ≤25-year-old group. Knowledge about HPV, HPV infection and its association with human diseases is poor in both groups. Only few participants know that HPV can cause benign and malignant oral lesions. After the information was given to the participants, higher knowledge scores were observed. This suggests that although these women have little knowledge about HPV and HPV vaccines, gaining more information may help them to understand more about these viruses. Public awareness and knowledge about HPV and HPV vaccine should be given to Thai women for a better prevention of HPV infection.

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Original Article

Tensile Bond Strength between Three Hard Reline Materials and Denture Base Resin Influenced by Methyl Formate-Methyl Acetate

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Abstract

This study investigated the effect of methyl formate-methyl acetate (MF-MA) wetting times on the tensile bond strength (TBS) between 3 non-MMA based reline materials and denture base material. Four hundred heat-cured denture base resin (Meliodent®) were prepared and randomly divided into 3 groups according to hard reline resins (Kooliner®, Tokuyama® Rebase II and Ufi Gel Hard®). Each group of reline material consisted of 6 or 7 subgroups (n=10), based on their surface treatment; control, adhesive, MF-MA 15, 30, 60, 180 s and MMA 180 s. The TBS test was performed using a Universal testing machine. Data were analyzed using one-way ANOVA and post hoc Tukey's analysis at $p<0.05$. The means TBS of the treated groups were significantly higher compared with those of the control group ($p<0.05$). In the Kooliner® groups, there were no significant differences in TBS between the MF-MA and the MMA treatment groups ($p>0.05$). In the Tokuyama® Rebase II groups, application of MF-MA solutions for 180 s produced the highest TBS compared with the other groups ($p<0.05$). In the Ufi Gel Hard®, the groups of MF-MA 180 s and MMA 180 s groups demonstrated significantly higher TBS compared with the other groups ($p<0.05$). Surface treatment with MF-MA solutions significantly increases the TBS between denture base resin and non-MMA hard reline resins. This study suggests that an MF-MA wetting time of 15 s for Kooliner® and 180 s for Tokuyama® Rebase II and Ufi Gel Hard® is adequate for creating a strong bond.

Keywords: Chemical surface treatment, Denture base, Methyl acetate, Methyl formate, Reline material, Tensile bond strength

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Introduction

Denture bases are made from poly (methyl methacrylate) resins which are constructed by connection of monomers to form polymer chain.[1] Fabrication of denture base has to bring about mechanical and physical properties, although poor fitting of the prosthesis always occur with the passing of time. This is because alveolar bone resorption is a continuous process due to tooth loss, causing denture base to be less stable on the ridge.[2] Therefore, dental prosthesis should be examined periodically and re-established to increase their adaptation. Relining a denture base with the reline materials is a common procedure to reproduce the fit of the denture and to improve the masticatory function.[3] Two main types of denture lining materials, classified by consistency, are soft and hard liners.[4] Soft liners are intended to be used for reducing masticatory force to the residual ridge. These liners consist of plasticizers, which serve as 'stress absorber' between denture and the underlying tissue.[5] However, prolonged exposure to water produces significantly higher hardness values and lower bond strength values.[6] Hard reline materials contain methyl methacrylate (MMA) or other type of monomers.[1] MMA can dissolve and penetrate into the denture base forming an adhesion.[7] After the setting of reline materials, residual monomers still leach out for a month causing oral tissue inflammation by direct contact.[8,9] Non-MMA based reline materials have a large amount of cross-linking agents added to a liquid part, which promotes greater transverse bending strength.[10] The interface of reline material-denture base resin depends on the ability of the monomers in the reline resins to diffuse and penetrate into the denture base, forming Inter-penetrating polymer networks (IPN).[11] Failure of adhesion promotes microleakage which enhances staining and bacteria accumulation.[11,12] Thus, surface treatment has been suggested to revise poor bonding.[13,14] Some studies reported that chemical surface treatment increased the flexural strength, while mechanical surface treatment had no effect.[15] Application of chemical agents dissolves the surface of denture bases and improves the diffusion of reline resin monomers to the denture base.[7,14] A mixture of MF-MA solution has been investigated in recent years, as it provides a high bond strength similar to Methyl methacrylate (MMA).[16] Considering a mixture of MF-MA solutions, a ratio of 25:75 (CU Acrylic Bond, Faculty of Dentistry, Chulalongkorn University, Bangkok, Thailand) significantly increases the bond strength between denture base resin and reline resin.[17] The effect of various MF-MA wetting times on the tensile bond strength between non-MMA based reline materials and denture base has not yet been studied.

The objective of this study was to evaluate the effect of various MF-MA wetting times on the tensile bond strength between three non-MMA based reline materials and a denture base resin. The first null hypothesis was that there were no significant tensile bond strength differences between non-MMA based reline materials and a denture base resin when different wetting times of MF-MA surface treatment were used. The second null hypothesis was that no significant variation would be noted in the tensile bond strength between non-MMA based reline materials and a denture base resin when different chemical surface treatments were used. The third null hypothesis was that types of non-MMA based reline materials did not significantly affect the tensile bond strength between non-MMA based reline materials and a denture base resin when the same chemical surface treatments were used.

Materials and Methods

The method of this study mainly followed ISO10139-2:2009(E).[18] Four hundred heat-cured acrylic resin (Meliodent®) plates were prepared (25±3 mm^2 and 3±0.5 mm thick) by investing in dental stone in dental flasks. The flasks were then pressed (2,000 kgf) for 1 hour. The specimens were polymerized at 74°C for 9

hours (as recommended by the manufacturer). The plates were finished with silicon carbide paper (P500, TOA, Thailand) using an automatic grinding and polishing unit (NANO2000, Pace Technologies, USA). A digital vernier caliper (500 series, Mitutoyo Corp., Japan) was used to verify samples' dimension after polishing. The plates were stored in a water bath (160M, Contherm Scientific Ltd., New Zealand) at 37±1°C for 28±2 days. The surface of each heat-cured acrylic plate was visualized using stereo microscope (SZ61, Olympus Corp., China) before receiving surface treatment. Next, the samples were randomly divided into three groups of hard reline materials [Group I: Kooliner® (n=60), Group II: Tokuyama® Rebase II fast (n=70), Group III: Ufi Gel Hard® (n=70)]. Each group consisted of six to seven subgroups (n=10), according to surface treatment (Fig. 1).

Table 1 *Trade name, manufacturer and chemical composition of the tested materials.*

Product name	Lot No. and Manufacturer	Composition		
		Powder	Liquid	Adhesive
Heat-activated acrylic resin (Meliodent®)	2018457, Tokuyama Dental Corp., Japan	PMMA	MMA	-
Self-cured hard reline (Kooliner®)	1211074, GC America, USA	PEMA	IBMA	-
Self-cured hard reline (Tokuyama® Rebase II Fast)	035EZ4, Tokuyama Dental Corp, Japan	PEMA	AAEMA 1,9 NDMA	Ethyl- acetate Acetone
Self-cured hard reline (Ufi Gel Hard®)	1511506, Voco, Germany	PEMA	1,6 HDMA	Acetone, 2-HEMA
Methyl Acetate	S6246689, Merck Schuchardt OHG, Germany	-	-	-
Methyl Formate	S6238911, Merck Schuchardt OHG, Germany	-	-	-

PEMA, poly(ethyl methacrylate; 1,6 HDMA, 1,6-hexanediol dimethacrylate
IBMA, isobutyl methacrylate; AAEMA, 2-(Acetoacetoxy) ethyl methacrylate
1,9 NDMA, 1,9-Nonanediol dimethacrylate; 2-HEMA, 2-Hydroxyethyl methacrylate

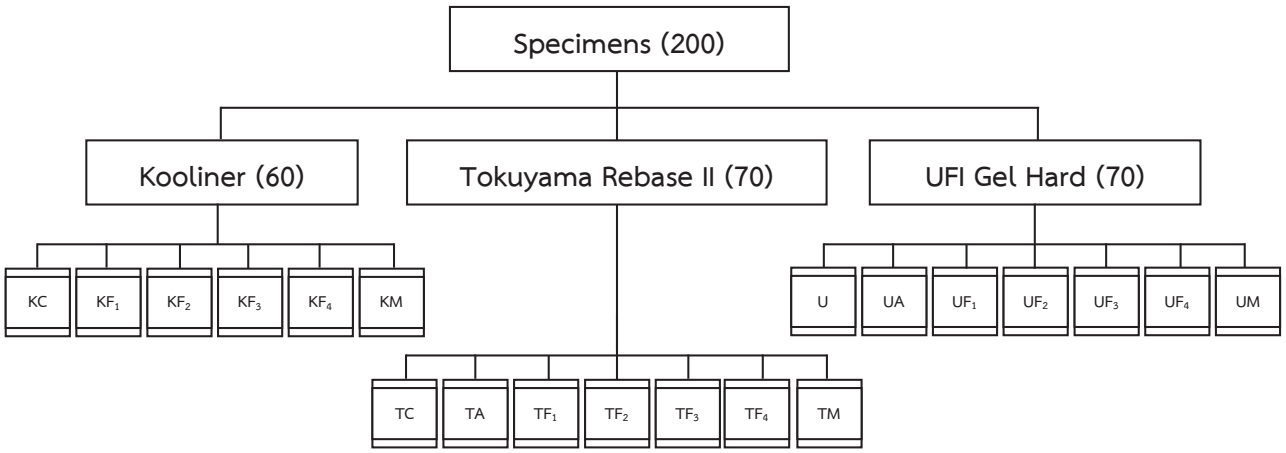

Figure 1 *The distribution of the specimens from each material. "K": Kooliner®, "T": Tokuyama® Rebase II fast, "U": Ufi Gel Hard®. "C": negative control groups (3 groups)-not treated with any solution on the bonding surface, only lined with the three reline materials. "M": MMA groups (3 groups)-treated with monomer of Meliodent® (MMA) monomer for 180 s. "A": adhesive was used following the manufacturer's recommendation in Tokuyama® Rebase II fast (T) and Ufi Gel Hard® (U) groups. A single layer of adhesive bonding agent had been applied before the reline material was loaded. Kooliner® did not require an adhesive bonding agent. "F": application of MF-MA solution for varying wetting times, 15 s(1), 30 s(2), 60 s(3), and 180 s(4), before applying the reline material.*

The specimens were constructed in a metal split mold (Fig. 2[B]) at room temperature. A bond area was controlled by Teflon collar; 10 mm diameter and 3 mm in height. Two plates of heat-cured acrylic resin that were separated by self-cured acrylic resin were used to form one test specimen. The test specimen was pressed by a 4 kg metal pendulum, simulating complete denture maximum bite force.[19] After the hard reline had set, the test specimens (Fig. 2[C]) were placed in a water bath at 37±1°C for 23±1 hrs. Two hundred test specimens were evaluated using a tensile strength testing machine in a vertical alignment (Fig. 2[E]). The tensile bond strength was measured by a Universal testing machine (8872, Instron Co., UK) with crosshead speed at 10 mm/min. The maximum load was recorded during debonding and the bond strength was calculated according to the following equation.

$$B = F/A$$

Where B was the tensile bond strength in MPa, F was the maximum load in Newton before debonding occurred and A was the adhesive area (mm^2).

The mode of failure of the debonded surface was determined (cohesive, mixed or adhesive failure) using a stereomicroscope (SZ61, Olympus Corp., China) at 10x magnification. Cohesive failure was defined as a failure where there was more than 50 % of the reline material on the denture base surface. Adhesive failure was defined as a failure where there was no trace of reline material on the denture base surface. Mixed failure was defined as a failure where there was less than 50 % of the reline material on the denture base surface.

Differential scanning calorimetry was conducted using a differential scanning calorimeter (DSC 7, Perkin-Elmer, Waltham, USA) to determine the exothermic energy of autopolymerizing hard reline materials. Each specimen of the hard reline materials was placed into an aluminum pan and the test was performed under a nitrogen purge with a flow rate of 70 mL/min. The scan speed for thermal heating was 10°C/min and the temperature range was from 25–120°C.

Data were analyzed using SPSS for Windows 25.0 (SPSS Inc., Chicago, IL, USA). The Kolmogorov Smirnov test was used to determine the normal distribution of the results and the equality of variance was evaluated using the Levene's test. The results did not conform to the assumptions of the Two-Way ANOVA that the data had to be statistically independent and with an equal number of observations. There is an interaction effect on the tensile strength between the two factors of the hard reline materials and the surface treatments. The interaction effect between two factors is defined as one in which the effect of one factor depends on the level of the other factor.[20] Thus, the results were statistically analyzed by One-Way ANOVA and Tukey HSD test.

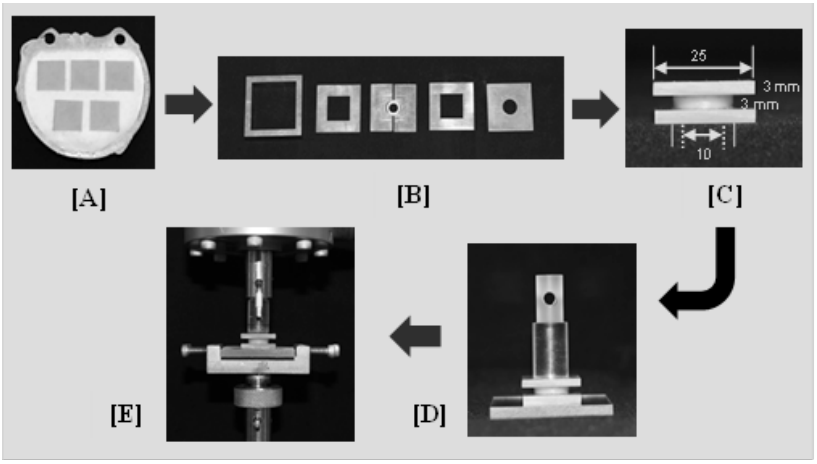

Figure 2 *Specimen preparation. [A] heat-cured denture base in a dental flask, [B] split metal mold, [C] test specimen, and [D,E] test specimen in a vertical alignment.*

Results

The mean tensile bond strength and standard deviation of each group and the percentage of each failure type were presented in Table 2. The mean tensile bond strength of the treated groups were significantly higher than those of their respective control groups ($p<0.05$). In the Kooliner groups, there were no significant differences in the tensile bond strength between the MF-MA solution wetting time groups and the MMA 180 s group ($p>0.05$). The tensile bond strength of the Tokuyama rebase II groups showed that the groups applied with MF-MA solution for 15, 30 and 60 s were not significantly different from that of the adhesive and the MMA 180 s group ($p>0.05$). In the Ufi Gel Hard groups, there were no significant differences in the tensile bond strength between the groups applied with MF-MA for 15, 30 and 60 s and the adhesive group ($p>0.05$). However, the mean tensile bond strength of the MF-MA 15 s, 30 s, 60 s and the adhesive groups were significantly lower than those of the MF-MA 180 s and the MMA 180 s groups ($p<0.05$). The mean tensile bond strength of the MF-MA 180 s group was not significantly different from that of the MMA 180 s group (MU) ($p>0.05$).

Failure type analysis demonstrated that all 3 reline materials in the control groups had 100 % adhesive failure. Most of the failures in MF-MA and MMA groups were mixed failures. The Tokuyama Rebase II and the Ufi Gel Hard MF-MA 180 s groups presented 40 % and 10 % cohesive failure, respectively, whereas the Ufi Gel Hard MMA 180 s group demonstrated 20 % cohesive failure. The percentage of the failure types in each group was shown in Table 3. The failure patterns by stereo-microscopy images were shown in Figure 3, 4, and 5.

Table 2 *The mean tensile bond strength of each reline material according to surface treatments*

Surface treatment	Kooliner	Tokuyama Rebase II fast	Ufi Gel Hard
control	4.94 ± 0.75 ^{B}	3.04 ± 0.72 ^{A}	3.53 ± 0.79 ^{A}
Adhesive	-	5.17 ± 0.61 ^{B,C}	5.21 ± 0.80 ^{B,C,D}
MF-MA 15 s	7.38 ± 0.40 $^{E, F, G}$	5.81 ± 0.45 ^{B,C,D}	5.42 ± 0.77 ^{B,C,D}
MF-MA 30 s	7.82 ± 0.88 ^{G}	5.68 ± 0.52 ^{B,C,D}	6.19 ± 0.82 ^{C,D,E}
MF-MA 60 s	7.50 ± 0.64 ^{F,G}	5.28 ± 0.80 ^{B,C,D}	6.29 ± 0.70 ^{C,D,E}
MF-MA 180 s	7.98 ± 0.52 ^{G}	7.85 ± 0.79 ^{G}	7.83 ± 0.90 ^{G}
MMA 180 s	8.23 ± 0.53 ^{G}	6.40 ± 0.74 ^{D,E,F}	7.90 ± 0.72 ^{G}

The same superscript letter indicated no significant difference between groups ($p>0.05$).

Table 3 *The percentage of failure pattern of the three reline materials and different surface treatments*

Surface treatment	Kooliner			Tokuyama Rebase II			Ufi Gel Hard		
	Co (%)	Mixed (%)	Ad (%)	Co (%)	mixed (%)	Ad (%)	Co (%)	Mixed (%)	Ad (%)
control	-	-	100	-	-	100	-	-	100
Adhesive	-	-	-	-	100	-	-	50	50
MF-MA 15 s	-	90	10	-	100	-	-	80	20
MF-MA 30 s	-	80	20	-	90	10	-	90	10
MF-MA 60 s	-	90	10	-	90	10	-	100	-
MF-MA180 s	-	90	10	40	60	-	10	90	-
MMA 180 s	-	80	20	-	100	-	20	80	-

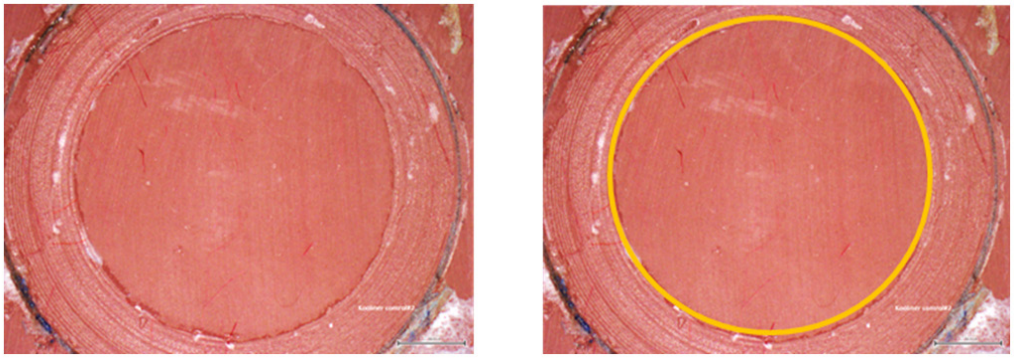

Figure 3 *Adhesive failure, no reline material attached, at denture base surface using a stereomicroscope at 10x magnification.*

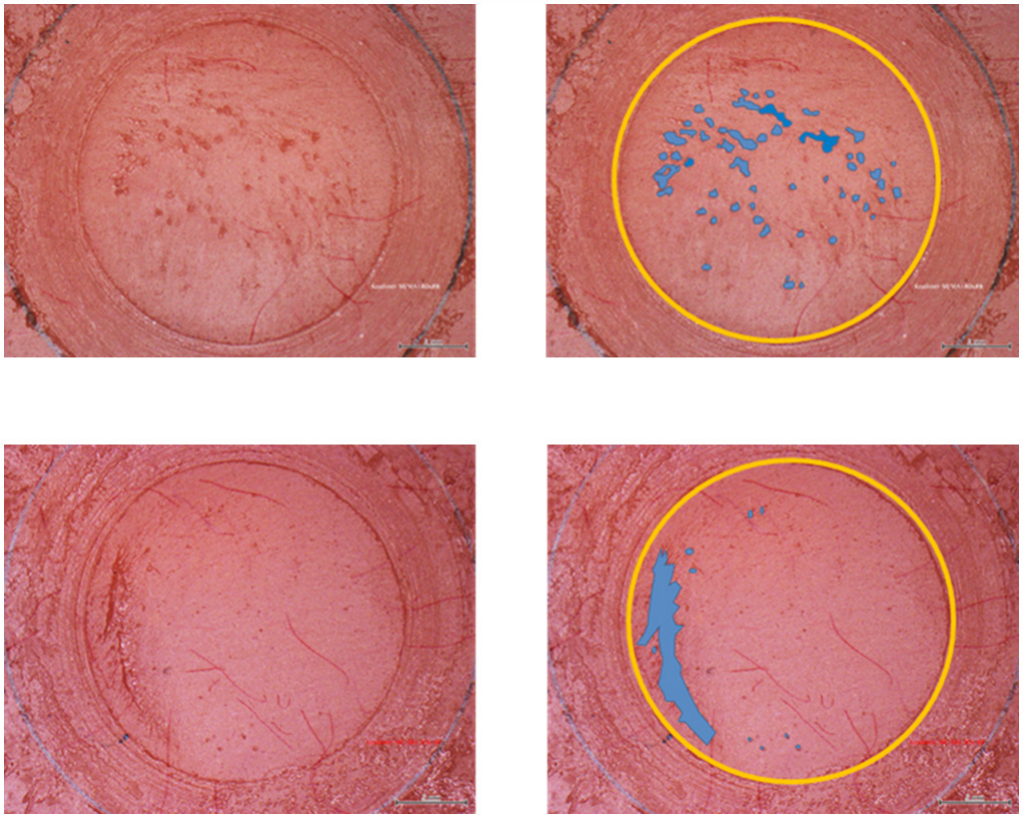

Figure 4 *Mixed failure showing the reline material (<50%) attached to the denture base surface using a stereomicroscope at 10x magnification. Blue area in the right representing the reline material.*

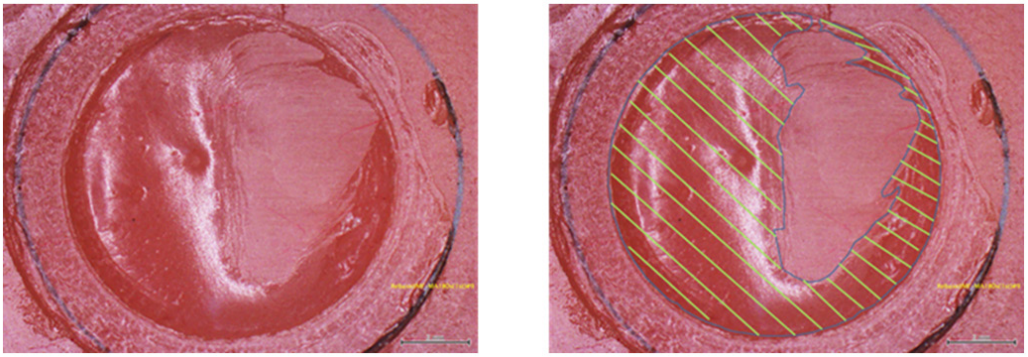

Figure 5 *Cohesive failure showing most of the reline material (>50%) attached to the denture base surface using a stereomicroscope at 10x magnification. Shading area in the right representing the reline material.*

SEM examination was used to observe the morphological changes on the denture base surface after surface treatment (Fig. 6). The untreated denture base surface, and the control group, exhibited scratch lines in a single direction with some acrylic debris from polishing (Fig. 6[A]). The surface of the denture resin treated with MF-MA for 15 and 30 s demonstrated numerous porosities with different sizes and patterns in the superficial layer, however, the deep layer still showed scratch lines (Fig. 6[B,C]). Denture base resin applied with MF-MA for 60 s showed the same surface pattern as the 15 and 30 s wetting times and with obscured scratch lines in the deep layer (Fig. 12[D]). The denture base resin treated with MF-MA for 180 s demonstrated a honeycomb appearance with 3-dimensional pores from the superficial into the deep layer (Fig. 6[E]). The denture base resin treated with MMA for 180 s had irregular scratch lines similar to the denture base resin applied with Tokuyama Rebase II adhesive (Fig. 6[F,G]). The Ufi Gel Hard adhesive created a smoother denture base surface (Fig. 6[H]).

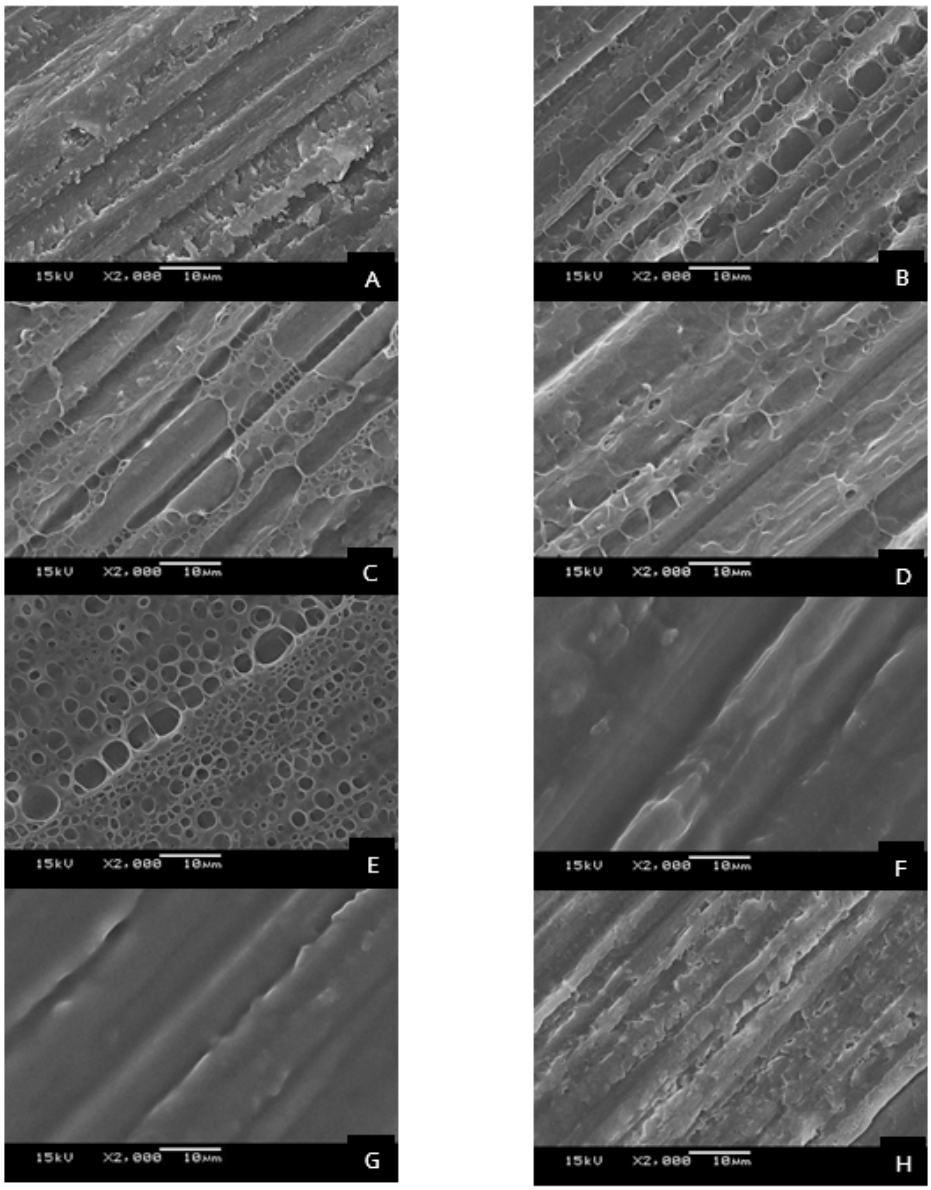

Figure 6 *SEM analysis of the morphological changes of heat-cured denture base surface treated with different surface treatments. [A] no treatment, [B] MF-MA solutions 15 s, [C] MF-MA solutions 30 s, [D] MF-MA solutions 60 s, [E] MF-MA solutions 180 s, [F] MMA 180 s, [G] Tokuyama Rebase II adhesive, [H] Ufi Gel Hard adhesive, respectively.*

Discussion

This study was designed to determine how various MF-MA wetting times affected the tensile bond strength between 3 non-MMA based reline materials and the denture base resin. The tensile bond strengths of specimens treated with MF-MA for 15, 30, 60, 180 s and no treatment were compared. These wetting times were selected based on a previous study that found that increased MMA wetting time caused an increased thickness of the swollen layer at the denture base surface.[21] Vallittu *et al.* concluded that an MMA wetting time of 180 s was sufficient to provide a strong bond.[7] Therefore, we used MF-MA wetting times ranging from 15-180 s to determine the optimum time for the highest tensile bond strength.

There are two main variables which directly relates to the tensile bond strength, reline materials and the surface treatment. Surface treatment refers to two factors, type of solvent and wetting time.

In the Kooliner groups, there were no significant differences in the tensile bond strength among the various MF-MA wetting times. However, the mean tensile bond strengths of the Tokuyama Rebase II and Ufi Gel Hard were significantly different in 180 s-MF-MA wetting time compared to those of the 15, 30 and 60 s-MF-MA groups. The mean tensile bond strengths of the Tokuyama Rebase II and Ufi Gel Hard of the 15, 30 and 60 s-MF-MA groups were not significantly different from each other. From the two-dimension appearance from 180 s-MF-MA SEM image (Fig. 6[E]), it was postulated that the monomer of Tokuyama Rebase II and Ufi Gel Hard could penetrate and form the better bond compared with 15, 30 and 60 s-MF-MA wetting time. The first null hypothesis was rejected.

Four solvents were used for denture base surface treatments (MF-MA, MMA, Ufi Gel Hard adhesive and Tokuyama Rebase II adhesive). The Ufi Gel Hard adhesive contains 2-HEMA and acetone, whereas the Tokuyama Rebase II adhesive includes ethyl acetate and acetone. The dissolution efficiency can be explained by the relative closeness of solubility parameters and polarities of PMMA and the solvents.[22] The solubility parameter of PMMA is 18.3 MPa½, while those of MF, MA, MMA, ethyl acetate and acetone are 20.9, 19.6, 18.0, 18.2 and 19.7 MPa½, respectively.[23] The solubility parameter of 2-HEMA (26.93 MPa1/2) is markedly different from that of PMMA. The MF, MA and MMA have similar polarities due to their methyl ester groups that enhance their ability to soften PMMA, while the other solvents have different functional groups. Acetone has ketone group. Ethyl acetate is ethyl ester. 2-HEMA contains ethyl ester and hydroxyl group. The dissimilar polarity of ethyl acetate, acetone and 2-HEMA to PMMA is likely to bring these compounds out of the range of effective solubility.[22]

The molecular weight of solvent has an effect on the softening efficacy, in which lower molecular weight promotes the faster kinetics of diffusion.[22] Acetone (58.08 Da) has a molecular weight close to MF (60.05 Da). The other four solvents have the higher molecular weight; MA (74.08 Da), ethyl acetate (88.11 Da), MMA (100.12 Da) and 2-HEMA (130.14 Da) than acetone and MF. Boiling point affects the bonding process in that lower boiling point of solvent causes an easier evaporation and takes less chair-time. Methyl formate (31.8°C) has the lowest boiling point compared to the other solvents. Methyl acetate (56.9°C) and acetone (57°C) have a similar boiling point. Ethyl acetate, MMA and 2-HEMA have a boiling point of 77.1°C, 101°C and 213°C, respectively.

As in the aforementioned, MF and MA have a low boiling point, 31.8°C and 56.9°C, respectively, compared to the other solvents.[24,25] This allows the solution to evaporate with no residual on the bonding surface after their application. The bond mechanism between 2 materials has two processes, diffusion and penetration.[26] First, the solvent diffuses and adheres to the denture base surface. This process is related to the size of the solvent molecules.[26] MF and MA have smaller molecules compared with MMA and the other two adhesives. The

second process is dissolution and penetration. MF-MA solution generates a swollen gel-like pattern on the denture base surface. This process depends on the solubility parameter, polarity and the concentration of the solvent in the polymer.[27] The similar solubility parameter and polarity of MF-MA compared to PMMA are one of the reasons for providing a good bond at the relined interface. The molecular structures of MF and MA also do not contain carbon–carbon double bonds (C=C) that might polymerize with the monomer of the autopolymerized reline materials. Thus, using MF-MA solution can create a proper bond area without any residual material that can block the bonding. The large amount of pores at the interface of the MF-MA treated relined denture base surfaces allow the monomer of the reline material to penetrate, and then polymerize to create a mechanical interlocking bond at the molecular level. Subsequently, an interpenetrating polymer network layer is formed between the denture base and the reline material.

Methyl methacrylate is a solvent commonly used for the surface treatment. This solvent has similar solubility parameter and polarity compared to PMMA. However, a higher molecular weight and boiling point of MMA might provide a lower solubility to the denture base material compared to MF-MA. Ethyl acetate and acetone have similar solubility parameter compared to PMMA, but they have different functional groups in their chemical structures. Besides, ethyl acetate has a higher molecular weight and boiling point compared to MF-MA and acetone. Acetone has many requirements to promote PMMA dissolution similar to MF-MA except the different functional group in chemical structure. 2-HEMA has not only a considerably higher molecular weight and boiling point compared to the other solvents, but also dissimilar solubility parameter and polarity. Thus, it would explain why 2-HEMA is not a good effective promotor to dissolve PMMA. The second null hypothesis was rejected.

The mean tensile bond strength of the Kooliner groups was significantly higher compared with those of the Tokuyama Rebase II and the Ufi Gel Hard groups. The molecular weight of the liquid part of reline materials plays a role in its viscosity. The Tokuyama Rebase II liquid contains AAEMA (214.21 Da) and 1,9 NDMA (296.40 Da) that are higher in molecular weight compared with the IBMA (142.20 Da) in the Kooliner, or the 1,6 HDMA (254.32 Da) in the Ufi Gel Hard. According to the diffusion theory, the higher the viscosity, the slower the material moves.[28] The high molecular weight of the components of the liquid monomer of Tokuyama Rebase II and Ufi Gel Hard retards the diffusion reaction in the polymerization process. Differential scanning calorimetry was used to calculate the exothermic energy of the 3 reline materials after mixing until the complete setting. The released energy of Kooliner, Tokuyama Rebase II and Ufi Gel Hard were 179.7, 121.7 and 150.5 J/g, respectively (Fig. 7). The heat generated during polymerization stimulates the rate of diffusion of the monomer molecules into the denture base material, enhancing the tensile bond strength. These two reasons, molecular weight of monomer and exothermic energy, account for the higher tensile bond strength of the Kooliner compared to other materials. The third null hypothesis was rejected.

For the failure patterns of specimens, the amount of tensile bond strength is positively related to the type of failure observed. From the correlation analysis, the higher tensile bond strength tends to be cohesive failure more than the mixed or the adhesive failure. However, this analysis cannot be applied to the relation between the mean tensile bond strength and the failure pattern in Kooliner group. The Kooliner group had a higher mean tensile bond strength compared with the two other materials, however, this group only exhibited mixed and adhesive failures. Previous studies have found in the same way with the failure result of this study that adhesive failure was generally occurred in the Kooliner specimens.[29-34] The Tokuyama Rebase II groups showed mixed and adhesive failures for all treatments except for the MF-MA 180 s group that showed cohesive failure. The Ufi Gel Hard groups showed all three failure types with cohesive failure in the MF-MA 180 s and the MMA 180 s groups. The non-harmonized mixing and the

powder-liquid ratio of the Tokuyama Rebase II and the Ufi Gel Hard might affect the failure results of these two materials by possibly creating voids in the reline materials. Once the test specimens were applied on the tensile force, it would be broken at the weakest area, sometimes at the void in the reline material.

Further researches using the flexural strength test, similar to the oral cavity condition, and thermocycling condition are required to confirm the effect of MMA and MF-MA solution on the bond strength between hard reline materials and a heated-polymerized acrylic denture base.

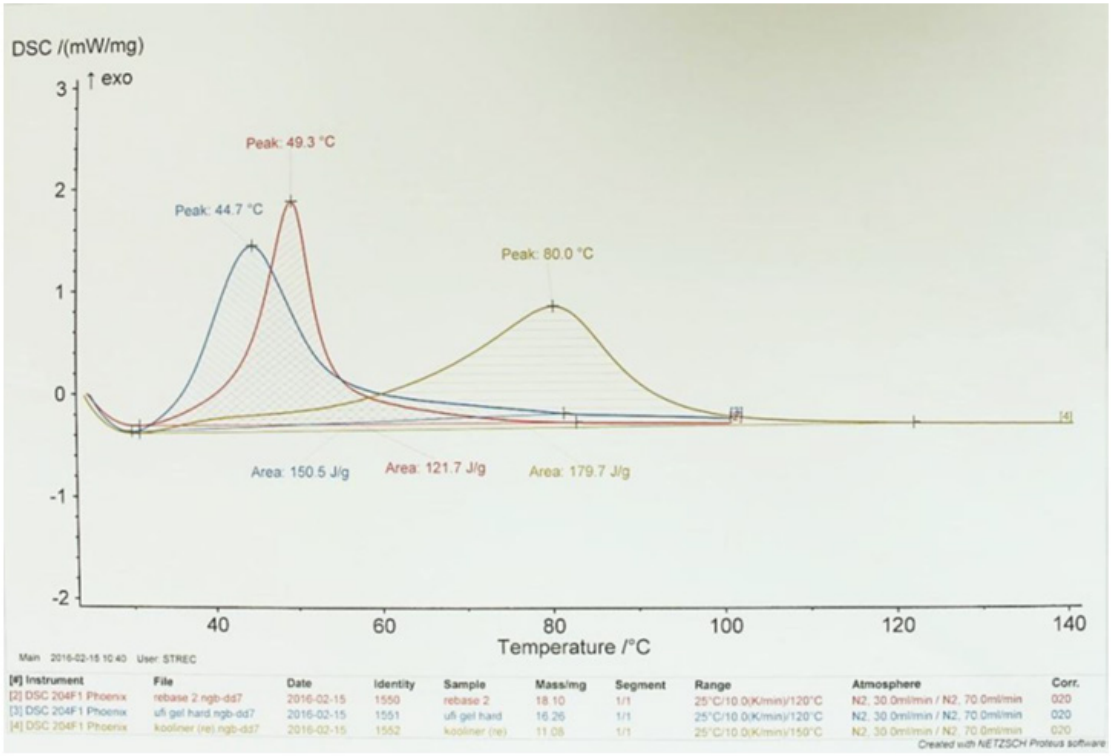

Figure 7 *Differential scanning calorimetry (DSC) analysis of each reline material (Kooliner in yellow line, Tokuyama Rebase II in red line and Ufi Gel Hard in blue line)*

Conclusion

Surface treatment with MF-MA solutions significantly increases the tensile bond strength between denture base resin and non-MMA based hard reline resins. This study suggests that a 15 s-MF-MA wetting time is adequate for creating a strong bond when using Kooliner as a reline material. MF-MA at a 180 s wetting time significantly enhances the tensile bond strength of the Tokuyama Rebase II fast and Ufi Gel Hard reline materials, and also reduces adhesive failure at the relined interface.

Clinical suggestions arising from this research

MF-MA solution is a solvent of choice in the surface treatment prior to relining denture base surface with a hard reline material.

Acknowledgments

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Original Article

Effect of Various Pressure Cooker Curing conditions on Flexural Strength of Denture Hard Relining Materials

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Abstract

The aim of this study was to evaluate the effect of various curing conditions in a pressure cooker on the flexural strength of hard chairside reline resins. One hundred and forty hard chairside reline resin (Unifast™ Trad and Tokuyama® rebase II Fast) specimens were prepared per ISO 20795-1 (2013) and divided into 14 groups. Each material was cured following the manufacturer's instructions as a control group and six experimental groups: cured under 1,500 mmHg air or nitrogen compressed pressure cooker at 55°C for 10, 15 or 20 minutes. The specimens were stored in water at 37±1 °C for 50±2 hours before testing. The three-point bending test was performed using a universal testing machine at a cross-head speed of 5 mm/min. One-way ANOVA and post hoc Tukey's analysis at a 95% confidence level were used to statistically compare the mean flexural strengths of the groups. For each material, the flexural strength of the air and nitrogen compressed groups were significantly higher compared with the control group ($P<0.05$). The flexural strength of the 10-min nitrogen group was significantly higher compared with the 10-min air group ($P<0.05$). There was no significant difference in flexural strength between the 15-min nitrogen and 15-min air groups ($P>0.05$). However, the flexural strength of the 20-min nitrogen group was significantly higher compared with the 20-min air group ($P<0.05$). The flexural strength in the 10, 15, and 20 min curing time groups of each reline material with the same curing environment in the pressure cooker were not significantly different ($P>0.05$). Under the same curing conditions, Unifast™ Trad had significantly higher flexural strength compared with Tokuyama® rebase II ($P<0.05$). Curing in the pressure cooker increased the flexural strength of the hard chairside reline resins. Moreover, using nitrogen gas pressure with satisfactory curing duration increased the flexural strength compared with using air pressure.

Keywords: Acrylic resin, Flexural strength, Hard reline resin, Nitrogen gas, Pressure cooker

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Introduction

Following tooth extraction, the residual alveolar ridge undergoes bone remodeling that results in bone resorption. The residual alveolar ridge reduction occurs rapidly and continuously during the first six months to two years after tooth extraction.[1] Bone resorption leads to a poor fit of the removable denture base that is used to replace the extracted teeth, and also adversely affects the patient's speech and mastication. When the residual ridge is reduced, the denture base needs to be relined to restore its fit to the residual ridge, and to improve the support, retention and stability of the denture base.[2,3] When using the direct relining technique, an auto polymerized hard reline resin is directly relined on the denture base in the mouth. This technique is inexpensive, easy to perform, not time consuming and can be done in a single visit.[2] In addition, auto polymerized hard reline resins have demonstrated adequate physical and mechanical properties. The indications for using an auto polymerized hard reline resin are a poorly adapted prosthesis, poor retention and stability at delivery and after use, and found that the denture loss their properties.[4]

Hard chairside reline resins are classified as type 2 (auto polymerized resin) class 1 (powder and liquid) denture base polymer. Their polymerization initiates with a chemical reaction and do not require persistence of temperatures above 65°C to complete polymerization.[5] The initiation reaction is a redox reaction. The benzoyl peroxide initiator is activated by a reducing agent such as dimethyl-p-toluidine producing a reactive center (or free radical). In the present study, the flexural strength of hard chairside reline resins were investigated using two brands which are commercially available in Thailand, Unifast™ Trad (MMA based) and Tokuyama® rebase II Fast (non-MMA based).

Auto polymerized hard reline resins can be divided into two groups based on the composition of the main liquid constituents: MMA based and non-MMA based reline materials. The MMA based materials which are commercially available include Unifast™ Trad (GC Corp., Tokyo, Japan), Probase Cold® (Ivoclar, Liechtenstein) and Palapress Vario® (Heraeus Kulzer, Wehrheim, Germany). MMA based relining materials have better adhesion to denture bases due to MMA monomer that dissolve and penetrate into the denture base forming the interpenetrating polymer networks (IPN) which bonds the two layers of materials.[6,7] There is also higher flexural strength compared with non-MMA based reline materials.[8] Despite these advantages, MMA-based hard relines can irritate the oral mucosa due to residual MMA or exothermic heat during polymerization.[9] These problems have been resolved with the introduction of non-MMA based hard reline materials. The non-MMA based materials available on the market include Kooliner® (Coe Laboratories, Chicago, USA), Ufi gel hard® (Voco, Cuxhaven, Germany) and Tokuso Rebase II® (Tokuyama Dental Corp, Tsukuba, Japan). Non-MMA based materials contain high molecular weight methacrylate, such as ß-methacryloyl oxyethyl propionate (MAOP) and 1,6-Hexanedial dimethacrylate (1,6-HDMA monomers). The molecular weights of the MAOP and 1,6-HDMA monomers are 186 g/mol and 254 g/mol, respectively. These monomers are almost twice the molecular weight of the MMA monomer (approximately 100 g/mol). The usage of these monomers in the reline resin improve the material by reducing tissue irritation and heat generation during manipulation.[10] In addition, they contain cross-linking agents in their liquid constituents, which improve their transverse bending strength.[11]

Many studies found that one of the disadvantages of auto polymerized acrylic resin is residual monomer or unreacted methyl methacrylate (MMA). Residual monomer can affect on their mechanical properties such as flexural strength[12], reduce the glass transition temperature[13], increase the possibility of deformation of the material[14], limit tensile strength, increase water absorption[15,16] and also cause an allergic reaction[17], irritation and inflammation of the oral tissues.[4] Thus, various methods have been used to decrease the amount of residual monomer, such as immersing the relined denture in hot water (50-55°C)[18], immersing in water for 24 hours after complete polymerization[19], microwaving post-polymerized radiation[20],

and ultrasonic immersion in water[21-23] or ethanol solution.[24,25] Furthermore, some mechanical properties of auto polymerized hard reline resins can be increased by applying pressure to the curing environment.[26] The use of a pressure cooker can reduce the dimensional change and altered occlusion that occurs during auto polymerized acrylic resin polymerization.[27] In addition, less porosity and higher flexural strength of auto polymerized hard reline resin was observed after curing in a pressurized environment.[28]

Free radical addition polymerization is inhibited or retarded by higher oxygen concentrations.[29] Some studies also found that oxygen inhibits the polymerization of acrylic resin.[30] However, a comparative study of the effect of using nitrogen gas in a pressure cooker curing method on the flexural strength of auto polymerized hard reline materials has not yet been reported.

The aim of this study was to evaluate the effect of various curing conditions in a pressure cooker on the flexural strength of hard chairside reline resins. The first null hypothesis was that there is no significant difference in the flexural strength between the groups of hard chairside reline resins cured in a pressure cooker and the groups cured in room atmosphere. The second null hypothesis was that the flexural strength between the groups of hard chairside reline resins cured in a nitrogen pressure cooker and groups cured in an air pressure cooker are not significantly different. The third null hypothesis was that variation in the curing time in the pressure cooker does not significantly affect the flexural strength of hard chairside reline resins. The fourth null hypothesis was that the flexural strengths of the various hard chairside reline resins cured at the same condition are not significantly different.

Materials and methods

Seventy specimens were prepared from Unifast™ Trad in a stainless-steel mold (Fig. 1) with dimension of 64x10x3.3 mm (Fig. 2) following the manufacturer's instructions (Table 1) using seven different curing conditions. For the control groups, the specimens were cured at room temperature (25+1°C) and pressure (760 mmHg). For the air groups, a pressure cooker (Fig. 3) (IMT; Pressurepotter 003, Inmotion technology limited, Thailand) was connected to an air pump. (Fig. 4(a)) The pressure cooker was filled with water below the specimen level and the temperature control panel was adjusted to 55°C. The stainless-steel mold containing the material was placed on a stand inside the pressure cooker and the lid was closed tightly, and compressed with 1,500 mmHg air (2 bar). The specimens were cured for 10, 15 or 20 minutes. For the nitrogen groups, the pressure cooker was connected to a nitrogen tank via a polyurethane tube. (Fig. 4 (b)) The tip of the polyurethane tube was placed under the water level. After adjusting the temperature to 55°C, the nitrogen valve was opened to let nitrogen gas flow into the pressure cooker. The pressure release valve of the pressure cooker was opened simultaneously to purge the air with nitrogen gas for five seconds. The pressure release valve was closed to rise the pressure to 1,500 mmHg (2 bar). The specimens were cured under nitrogen gas pressure for 10, 15 or 20 minutes. Another 70 specimens were prepared from Tokuyama® rebase II using the same procedures as described for Unifast™ Trad. The cured Tokuyama® rebase II specimens were soaked in a Hardener® water solution (40-60°C) for three minutes then rinsed and dried. The 140 specimens were distributed into 14 groups (n=10) based on their curing condition.

The specimens were polished with metallographic grinding paper (P500, TOA, Thailand), on a polishing machine (NANO2000, Pace Technologies, USA) by wet grinding on both sides to a 3.3 mm thickness. The specimens were stored in water at 37±1°C for 50±2 hours prior to flexural strength testing. The specimens were removed from water storage and immediately subjected to the flexural strength test, following ISO 20795-1:20135, using a 3-point loading universal testing machine (SHIMADZU; EZ-S, SHIMADZU, JAPAN) at a cross-head speed of 5 mm/min, a span of 50 mm, and 500 N load cell until the specimen broke. (Fig. 5) The flexural strength (MPa) was calculated using the following equation:

$$\sigma = \frac{3Fl}{2bh^2}$$

σ = the load (N) at fracture
l = the distance between supports (mm)
b = mean specimen width (mm)
h = mean specimen height (mm)

The normality of the flexural strength data of each group was determined by using the One-Sample Kolmogorov-Smirnov test and the variance was evaluated using the Levene test. If the data had a normal distribution with equal variance, two-way analysis of variance (Types of reline materials, and pressure cooker curing conditions), with a 95% confidence level, was used to determine the significance. If the results did not conform to the assumptions of two-way ANOVA that the data had to be statistically independent and with an equal number of observations, one-way analysis of variance (post hoc Tukey with a 95% confidence level), was used to determine the significance.

Table 1 *Chemical composition and manufacturer of the materials.*

Material	Major ingredients	Mixing time	Working time	Powder-liquid ratio	Manufacturer
Unifast ™ Trad	Powder: PMMA, MMA&EMA copolymer Liquid: MMA monomer, dimethyl-p-toluidine	10-15 sec	2 mins	1.0g / 0.5mL	GC Corporation, Tokyo, Japan
Tokuyama® Rebase II Fast	Powder: PEMA Liquid :1,9-NDMA, AAEMA Hardener®: Sodium bicarbonate, Sodium sulphite	5-10 sec	20 -60 sec	2.40g / 1.0mL	Tokuyama dental corporation, Tokyo, Japan

PMMA, Poly (methyl methacrylate); MMA&EMA copolymer, Methyl methacrylate & Ethyl methacrylate copolymer; PEMA, Poly (ethyl methacrylate); 1,9NDMA, 1,9nonanedioldimethacrylate; AAEMA, 2-(acetoacetoxy) ethyl methacrylate.

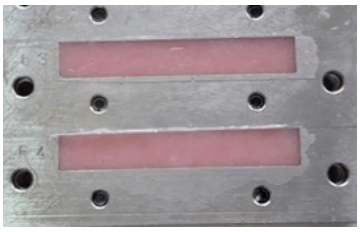

Figure 1 *Stainless steel mold with loaded material*

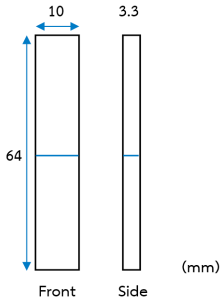

Figure 2 *Illustration of the 64x10x3.3 mm specimens*

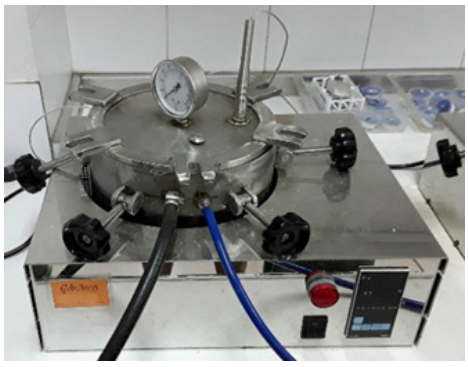

Figure 3 *Pressure cooker with pressure release valve on the top surface of the lid. The temperature control panel is on the front side of the set up.*

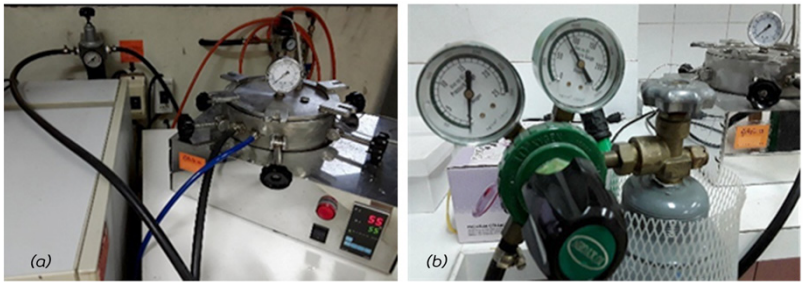

Figure 4 *(a) pressure cooker connected with air pump, (b) pressure cooker connected with nitrogen gas.*

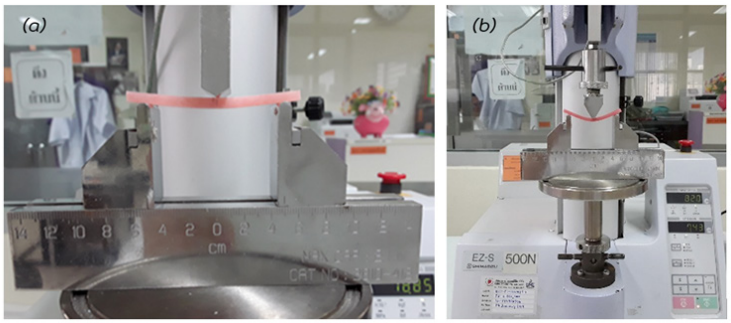

Figure 5 *(a) lay the flat surface symmetrically on the supports of the flexural test rig, (b) flexural strength test, using 3-point loading universal testing machine.*

Results

The data were analyzed using the Kolmogorov-Smirnov test to determine data distribution. The results indicated that the data were normally distributed in all groups (P>0.05). The results did not conform to the assumptions of two-way ANOVA that the data had to be statistically independent and with an equal number of observations (Table 2). The interaction effect between the two factors (types of reline materials and pressure cooker curing conditions) is defined as one in which the effect of one factor depends on the level of the other factor. Thus, the results were statistically analyzed by one-way ANOVA and Tukey HSD test.

Table 2 *Two-way ANOVA analysis of the mean flexural strength.*

Source	Type III Sum of Squares	df	Mean Square	F	*P*
Corrected Model	20704.682[a]	3	6901.561	1065.669	.000
Intercept	473425.350	1	473425.350	73101.526	.000
product	20248.033	1	20248.033	3126.495	.000
atmosphere	406.014	1	406.014	62.693	.000
product * atmosphere	50.635	1	50.635	7.819	.006
Error	751.248	116	6.476		
Total	494881.280	120			
Corrected Total	21455.930	119			

The mean flexural strength and standard deviation of each group is presented in Table 3 and Figure 6. For each hard reline material, the flexural strength of the pressure cooker cured groups was significantly higher compared with the control group (P<0.05). For each hard reline material cured in the same environment in the pressure

cooker, the flexural strengths of the 10, 15, and 20 min curing time groups were not significantly different (P>0.05). Comparing the groups by curing time, the flexural strength of the 10-min nitrogen group was significantly higher than that of the 10-min air group (P<0.05). In contrast, no significant difference was found in flexural strength between the 15-min nitrogen and 15-min air group (P>0.05). However, the flexural strength of the 20-min nitrogen group was significantly higher compared with the 20-min air group (P<0.05). In addition, at the same curing conditions, Unifast™ Trad demonstrated a significantly higher flexural strength compared with Tokuyama® rebase II (P<0.05).

Table 3 *Mean flexural strength with standard deviation of the different groups.*

Curing conditions					
Pressure (mmHg)	Temperature (°C)	Curing environment	Curing time (min)	UT	TR+H
760	25	air	2(UT),5.5(TR)	66.40 (5.47) $^{A, a}$	45.98 (1.52) $^{A, b}$
1,500	55	air	10	72.42 (2.13) $^{B, a}$	48.35 (1.41) $^{B, b}$
1,500	55	air	15	73.16 (3.94) $^{BC, a}$	48.80 (1.85) $^{BC, b}$
1,500	55	air	20	73.93 (4.23) $^{BC, a}$	48.74 (1.81) $^{BC, b}$
1,500	55	N_2	10	77.92(2.72) $^{CD, a}$	50.69 (1.28) $^{CD, b}$
1,500	55	N_2	15	77.71 (2.82) $^{CD, a}$	50.89 (1.76) $^{CD, b}$
1,500	55	N_2	20	79.24 (4.27) $^{D, a}$	51.45 (1.29) $^{D, b}$

UT, Unifast ™ Trad, TR+H, Tokuyama® Rebase II Fast with Hardener

Same uppercase letter indicates no significant difference between the groups in each column (p>0.05)

Same lowercase letter indicates no significant difference between the groups in each row (p>0.05)

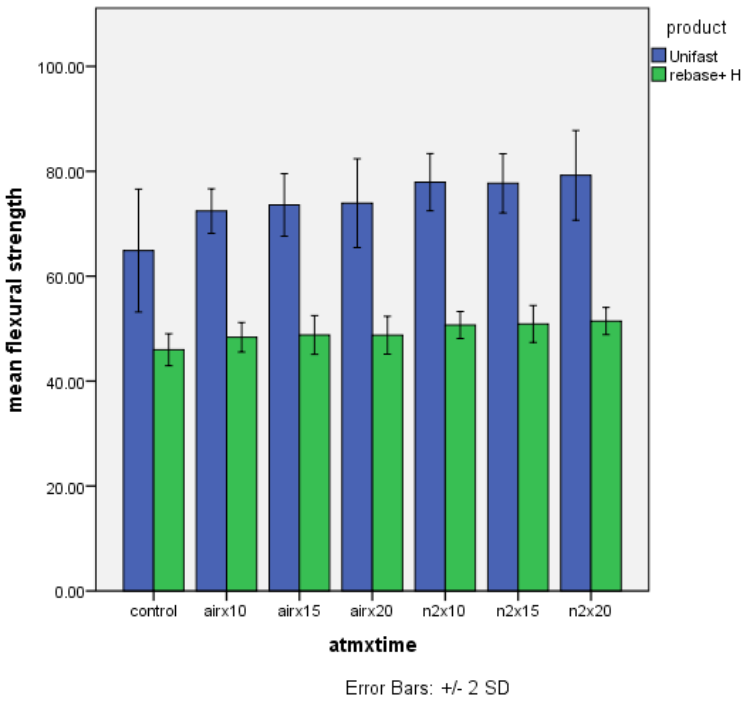

Figure 6 *The mean flexural strength (bars) of Unifast ™ Trad and Tokuyama® Rebase II Fast with Hardener at various conditions (MPa) and ± standard deviation (vertical lines) are given.*

Table 4 *P-values of One-way ANOVA analysis of UT and TR+H.*

Materials	Group comparison	Sig.
Unifast™ Trad	Control-Air 10 min	0.004
	Control-Air 15 min	0.002
	Control-Air 20 min	0.002
	Control-Nitrogen 10 min	0.000
	Control- Nitrogen 15 min	0.000
	Control- Nitrogen 20 min	0.000
	Air 10 min- Air 15 min	1.000
	Air 10 min- Air 20 min	1.000
	Air 10 min- Nitrogen 10 min	0.037
	Air 10 min- Nitrogen 15 min	0.032
	Air 10 min- Nitrogen 20 min	0.023
	Air 15 min- Air 20 min	1.000
	Air 15 min- Nitrogen 10 min	0.074
	Air 15 min- Nitrogen 15 min	0.064
	Air 15 min- Nitrogen 20 min	0.048
	Air 20 min- Nitrogen 10 min	0.075
	Air 20 min- Nitrogen 15 min	0.065
	Air 20min- Nitrogen 20 min	0.048
	Nitrogen 10 min- Nitrogen 15 min	1.000
	Nitrogen 10 min- Nitrogen 20 min	1.000
	Nitrogen 15 min- Nitrogen 20 min	1.000
Tokuyama® rebase II Fast	Control-Air 10 min	0.021
	Control-Air 15 min	0.003
	Control-Air 20 min	0.004
	Control-Nitrogen 10 min	0.000
	Control- Nitrogen 15 min	0.000
	Control- Nitrogen 20 min	0.000
	Air 10 min- Air 15 min	0.995
	Air 10 min- Air 20 min	0.998
	Air 10 min- Nitrogen 10 min	0.023
	Air 10 min- Nitrogen 15 min	0.011
	Air 10 min- Nitrogen 20 min	0.001
	Air 15 min- Air 20 min	1.000
	Air 15 min- Nitrogen 10 min	0.120
	Air 15 min- Nitrogen 15 min	0.062
	Air 15 min- Nitrogen 20 min	0.007
	Air 20 min- Nitrogen 10 min	0.100
	Air 20 min- Nitrogen 15 min	0.051
	Air 20 min- Nitrogen 20 min	0.005
	Nitrogen 10 min- Nitrogen 15 min	1.000
	Nitrogen 10 min- Nitrogen 20 min	0.933
	Nitrogen 15 min- Nitrogen 20 min	0.984

Discussion

This study was designed to determine how various pressure cooker curing conditions affect the flexural strength of denture hard relining materials. There are two main variables which directly relates to the flexural strength, curing conditions and reline materials. Curing conditions refers to three factors, pressure cooker, curing environment and curing time.

For each hard reline material, the flexural strength of the pressure cooker cured groups was significantly higher than that of the control group because of two factors, curing temperature and curing pressure. The curing temperature's effect on the rate and degree of polymerization is of prime importance in determining the manner of performing polymerization. Increasing the curing temperature usually increases the polymerization rate and decreases the percentage of residual monomer.[31] The effect of pressure on polymerization is important from the practical viewpoint because several monomers are polymerized at pressures that are above atmospheric pressure. High pressure can have appreciable effects on polymerization rates and polymer molecular weights. Increased pressure usually results in increased polymerization rates and molecular weight.[31] Increased pressure improved the flexural strength of the hard chairside reline resins when compressed with either air or nitrogen gas. Our results are consistent with those of previous studies that reported that auto polymerizing acrylic resin cured under pressure demonstrated decreased porosity and increase flexural strength.[28,32] The pressurized environment may prevent monomer evaporation during the initial stage of polymerization, thus, minimizing the pore formation and improving flexural strength.[33] The first hypothesis was rejected.

Because of the inert characteristics of nitrogen gas, it does not undergo chemical reactions. So, it is a consideration to use nitrogen as purging gas. Purging the pressure cooker and replacing with nitrogen gas may help increase the flexural strength of the hard chairside reline resins because oxygen exposure was eliminated. Oxygen is a powerful inhibitor, as demonstrated by the very large inhibition constant values (ratio of the rate constants for inhibition and propagation). This value of MMA polymerization is 33,000.[31] Oxygen reacts with radicals to form the relatively unreactive peroxy radical that reacts with itself or another propagating radical by coupling and disproportionation reactions to form inactive products (probably peroxides and hydroperoxides). Consistent with a study that reported that an excluded air curing environment decreased residual monomer.[30] Radical-chain polymerization can be inhibited by oxygen which reacts with free radicals. Large amounts of oxygen will compete with MMA for free radicals and inhibit polymerization. Oxygen has some characteristic like an unpaired electron, which can react with a free radical initiator or during propagation of polymer chain. Thus, the degree of inhibition is proportional to the concentration of oxygen.[31,33,34] However, a study found of unpolymerized layer of hard chairside reline resin after curing auto-polymerizing acrylic resin under pressure. It was assumed that higher air pressure might provide more oxygen to the resin surface and retard polymer chain growth and affect surface hardness.[35] The second hypothesis was rejected.

For each hard reline material cured at the same environment in the pressure cooker, the flexural strength of the 10, 15, and 20 min curing time groups were not significantly different. This may be explained by the termination of the degree of polymerization. The respective manufacturers recommend that the Unifast™ Trad setting time is two minutes, while that of Tokuyama® rebase II Fast setting time is 6-8 minutes. Thus, varying the curing time above these amounts might not affect flexural strength. Therefore, the present study assumes that curing under pressure, compressed with either air or nitrogen gas, at 1,500 mmHg 55°C for ten minutes results in a flexural strength equal to that of curing for 15 or 20 minutes. In addition, it should be noted that

handing that follows the manufacturers recommendation results in lower flexural strength due to less polymerization with room temperature, lower pressure, and oxygen exposure. The third hypothesis was accepted.

Under the same curing conditions, Unifast™ Trad had a significantly higher flexural strength compared with Tokuyama® rebase II. These results agree with those of previous studies.[8,36] The difference in flexural strength of the two types of reline materials might be due to the different molecular structures and mechanical properties of the polymerized materials. The Unifast™ Trad powder is composed of PMMA, while Tokuyama® rebase II mainly consists of PEMA. The Tokuyama® rebase II liquid consists of 59 % acetoacetoxy ethyl methacrylate (AAEM) monomer and 39 % 1,9-nonanediol dimethacrylate (1,9-NDMA) as the cross-linking agent.[37] The molecular weight of the AAEM monomer is 214.22 g/mol, and the cross-linking agent 1,9-NDMA has a higher molecular weight of 296.4 g/mol.[37] Unifast™ Trad predominantly consists of MMA monomer and dimethyl-p-toluidine, and the molecular weight of MMA is 100 g/mol.[38] In addition, because it is MMA-based, Unifast™ Trad has a higher exothermic behavior.[39] During polymerization proceeds, the carbon–carbon double bonds (C=C) are converted to carbon–carbon single bonds (C-C). The difference in energy between the two bonds may emit as heat.[40] The emitted heat increased the curing temperature and polymerization reaction. This resulted in higher flexural strength of Unifast™ Trad. Thus, the fourth hypothesis was rejected.

Conclusion

Within the limitations in this study, it can be concluded that curing in a pressure cooker significantly increases the flexural strength of auto polymerized hard reline resins. When performing curing in a pressure cooker, using nitrogen instead of air with appropriate curing time also significantly increases the flexural strength.

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Original Article

Effect of Methyl Formate-Methyl Acetate Treatment on Flexural Strength of Relined Denture Base

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Abstract

The purpose of this study was to evaluate the effect of methyl formate-methyl acetate (MF-MA) surface treatment on flexural strength between denture base and hard reline materials. One hundred heat-cured acrylic denture base (Meliodent®) specimens were prepared according to ISO 20795-1 (2013) and divided into ten groups. Groups I-III: relined with Unifast Trad®, Group IV-VI: relined with Kooliner® and Group VII-X: relined with Tokuyama® Rebase II Fast. Groups I, IV and VII were untreated surface (control groups), Groups II, V and VIII were surface treated with methyl methacrylate (MMA) for 180 s and Groups III, VI and IX were surface treated with methyl formate-methyl acetate (MF-MA) solution for 15 s, Group X were surface treated with the provided adhesive per the manufacturer's instructions. Flexural strength was measured using a Universal Testing Machine. The data were analyzed using two-way ANOVA (group I-IX) and one-way ANOVA (group I-X) where significant differences in the groups were found. The group means were compared using Tukey's test at a 95 % confidence level. The reline material type and surface treatments significantly affected on the flexural strength ($p<0.05$). For each reline material, the flexural strength of the MF-MA treated group was significantly higher compared with the other groups ($p<0.05$). For the same surface treatment, the flexural strength of Unifast Trad® was significantly higher compared with Kooliner® ($p<0.05$). The flexural strength of Kooliner® was higher than that of Tokuyama® Rebase II Fast ($p<0.05$). This study suggests the application of MF-MA solutions for 15 s before relining procedure can increase the flexural strength between the denture base and hard reline materials.

Keywords: Acrylic denture base, Flexural strength, Hard reline materials, Methyl formate-methyl acetate, MMA

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Introduction

Alveolar ridge, supported prosthesis, are continuously resorbed[1], resulting in loss of stability and tissue pain under prosthesis. Patients need to have their denture reline to restore good stability and retention.[2-4] The two methods for relining a denture base are direct and indirect relining. The indirect technique uses a heat-polymerizing resin in a laboratory, while the direct technique uses self-cured hard reline materials and is performed chairside. The direct technique is quick, easy and does not require laboratory procedures. Patients can use their prosthesis immediately after the relining is completed. However, the disadvantages of this method include reline odor and an unpleasant taste. This method can also cause tissue irritation under the denture base due to residual monomers and higher temperatures during polymerization.[5] Chairside hard reline procedures use self-cured hard reline materials to support increased tissue stability and retention. The classification of hard reline materials, based on the main component in the liquid, are MMA-based and non-MMA-based. MMA-based reline materials include Unifast Trad® (GC Corp., Tokyo, Japan), Probase Cold® (Ivoclar, Liechtenstein), and Palapress Vario® (Heraeus Kulzer, Wehrheim, Germany). Examples of non-MMA-based reline materials are Kooliner® (Coe Laboratories, Chicago, USA), Ufi gel hard® (Voco, Cuxhaven, Germany), and Tokuso Rebase II® (Tokuyama Dental Corp, Tsukuba, Japan). The non-MMA-based reline material monomers are higher molecular weight molecules that cause less tissue irritation. The MMA-based reline materials adapt well to the denture base; however, oral irritation can occur due to the residual monomer.[6-8]

Adhesion failure between the reline material and the denture base also causes the accumulation of bacteria and color change.[5,9,10] Adhesion failure also reduces the strength of the denture base.[5,9,11,12] There have been many techniques employed to increase the bond strength of the reline materials and denture base, such as grinding the denture base surface[13], using particles to create surface abrasion[14], and applying various chemical agents such as MMA[15-18], methylene chloride[15,19], chloroform[17], acetone[14,17], ethyl acetate[20], MF and MA[21,22]. However, chloroform and methylene chloride are carcinogens.[17] In addition, methyl methacrylate is irritating and can cause an allergic reaction.[23]

Vallittu *et al.,* 1994 concluded that MMA wetting time of 180 s was recommended to strengthen repaired acrylic resin.[18] Asmussen *et al.,* 2000 found that MF and MA surface treatment improved the shear bond strength between hard reline materials and denture base when using methylene chloride and compared to using ethyl acetate.[19] Thunyakitpisal *et al.,* 2011 found that the application of an MF-MA solution on the denture base surface for 15 s before doing repair work significantly increased flexural strength.[21] In addition, Osathananda and Wiwatwarrapan, 2016 also found that applying an MF-MA solution increased the shear strength between hard reline and denture base compared with using the adhesive recommended by the manufacturer.[22] A comparative study of the effect of the MF-MA surface treatment on flexural strength of the relined denture base has not yet been reported.

The objective of this study was to evaluate the effect of MF-MA surface treatment on flexural strength of relined denture base. The first null hypothesis was that the flexural strength of relined denture base groups with various chemical surface treatments were not significantly different from that of the untreated surface group. The second null hypothesis was that the flexural strength of relined denture base groups with various hard reline materials were not significantly different from each other. The third null hypothesis was that there was no significant difference in flexural strength between the relined denture base groups with various chemical surface treatments.

Materials and methods

The heat-cured acrylic denture resin, hard reline materials, and chemical agents used in this study are shown in Table 1. One hundred specimens of heat-cured acrylic denture base (Meliodent®) (64x10x2 mm) were prepared in a denture flask (Fig.1(a)) as recommended in ISO 20795-1 (2013).[24] The specimens were finished with 500-grit silicon carbide paper (TOA, Thailand) using an automatic grinding and polishing unit (Minitech 233, France) and then placed in a split metal mold (64x10x3.3 mm, Fig. 1(b)) and relined with their relining material (Fig. 1(c)).

The specimens were randomly divided into ten groups: Groups I, II and III were relined with Unifast Trad®; Groups IV, V, and VI were relined with Kooliner®; Groups VII, VIII, IX and X were relined with Tokuyama® Rebase II Fast. Groups I, IV and VII were the untreated surface control groups, Groups II, V and VIII were surface treated with Unifast Trad® (MMA) liquid for 180 s (by brush every five seconds) and then wait for 30 seconds to evaporate; Group III, VI and IX were surface treated with MF-MA solution (25:75 by volume) for 15 s (by brush every five seconds) and then wait for 30 seconds to evaporate; and Group X was surface treated with Tokuyama® Rebase II Fast adhesive following the manufacturer instructions.

The reline side of specimens were finished with a 500-grit new silicon carbide paper using an automatic grinding and polishing unit (Minitech 233, France) and stored in water at 37±1°C for 48±2 h. The flexural strength was measured by a universal testing machine (SHIMADZU, EZ-S 500N model, Japan) at a crosshead speed of 5 mm/min (Fig. 1 (d)). The flexural strength (MPa) was calculated using the following equation:

$$\delta = \frac{3Fl}{2bh^2}$$

Where δ = flexural strength (MPa)
F = the load (N) at fracture
l = the distance between supports (mm)
b = mean of specimen width (mm)
h = mean of specimen height (mm)

Table 1 *Types of materials and their manufacturers were used in this study*

Product name	composition	Manufacturer
Heat cured denture base		
Meliodent®	Powder:PMMA Liquid: MMA	Kulzer, German
Hard reline resins		GC America, USA
-MMA based	Powder:PMMA	
Unifast Trad®	Liquid: MMA	
-non-MMA based		
Kooliner®	Powder:PEMA Liquid:IBM	GC America, USA
Tokuyama® Rebase II Fast	Powder:PEMA Liquid: AAEMA, 1,9-NDMA	Tokuyama Dental Corp, Japan
Adhesive	ethyl acetate& acetone	
Chemical solvent		
Methyl formate		Merck Schuchardt OHG, German
Methyl acetate		Merck KGaA, German

PMMA, Poly(methyl methacrylate); MMA, Methyl methacrylate; PEMA, Poly(ethyl methacrylate); IBM, Isobutyl metacrylate; AAEMA, 2-(Acetoacetoxy) ethyl methacrylate; 1,9-NDMA, 1,9 Nonanediol dimethacrylate.

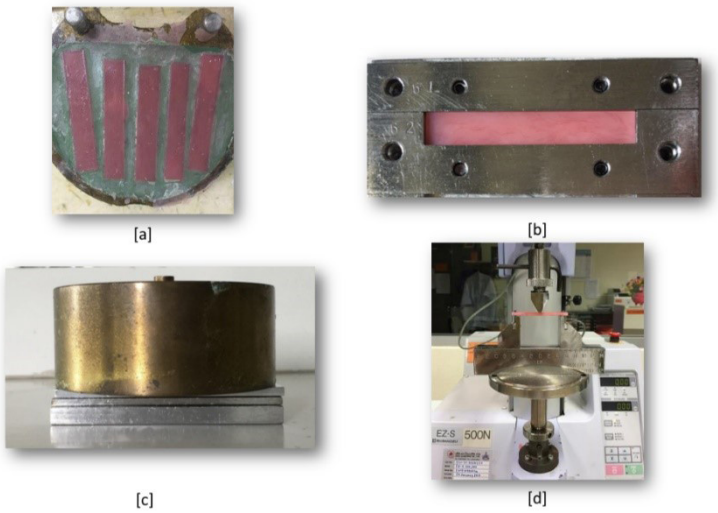

Figure 1 *(a) Heat-cured acrylic denture base (64x10x2 mm) specimens were prepared in a denture flask. (b) The specimens were placed in split metal mold (64x10x3.3 mm), applied with their respective surface treatment agent, and relined with a re lining material. (c) pressed lightly topped with 1 kg iron. (d) Flexural strength test.*

The data were analyzed using SPSS software version 22.0 (SPSS Inc., Chicago, IL, USA). The results were statistically analyzed by two-way ANOVA (group I-IX) and one-way ANOVA (group I-X) where significant differences in the groups in each row and each column were found, the group means were compared using Tukey's test at a 95% confidence level.

Result

The data were analyzed by using the Kolmogorov-Smirnov test to determine data distribution. The results showed that all data were normally distributed in all groups ($p>0.05$). The mean flexural strength and standard deviation of each group is presented in Table 2. The surface treatment and type of reline materials affected on the flexural strength ($p<0.05$) and the exact *P*-values are presented in Table 3 and Table 4.

For each material, the flexural strength of the surface treatment groups were significantly higher compared with the control group ($p<0.05$). The MF-MA treated group also had a significantly higher flexural strength compared with the MMA treated group for each hard reline material ($p<0.05$). However, in Tokuyama® Rebase II groups, there were no significant differences in the mean flexural strength between the groups treated with MMA or adhesive ($p>0.05$). For the same surface treatment, the flexural strength of Unifast® was significantly higher than that of Kooliner® ($p<0.05$), and the flexural strength of Kooliner® was significantly higher than that of Tokuyama® Rebase II ($p<0.05$).

Table 2 *The mean flexural strength with standard deviation of each reline material and surface treatment.*

Surface treatment	Reline materials		
	Unifast®	Kooliner®	Tokuyama® Rebase II
Control	79.56±2.35$^{a, A}$	72.28±2.47$^{a, B}$	60.05±2.45$^{a, C}$
MMA	88.94±3.72$^{b, A}$	76.42±3.18$^{b, B}$	64.60±2.22$^{b, C}$
MF-MA	97.53±2.36$^{c, A}$	81.09±2.17$^{c, B}$	71.97±2.48$^{c, C}$
Adhesive	-	-	65.95±2.57^{b}

****Same uppercase letter indicates no significant difference between the group in each row (p>0.05)*

****Same lowercase letter indicates no significant difference between the group in each column (p>0.05)*

Table 3 *Two-way ANOVA analysis of the mean flexural strength.*

Source	Type III sum of squares	df	Mean square	F	*P*
Corrected model	10757.685[a]	8	1344.711	192.297	< 0.005
Intercept	532774.108	1	532774.108	76187.921	< 0.005
Surface treatment	2497.784	2	1248.892	178.594	< 0.005
Reline materials	8032.067	2	4016.034	574.302	< 0.005
Surface treatment*reline materials	227.833	4	56.958	8.145	< 0.005
Error	566.424	81	6.993		
Total	544098.217	90			
Corrected total	11324.110	89			

Table 4 *The exact p-value in this study.*

Materials	Group comparison	Sig.
Unifast ®		
	Control- MMA	0.000
	Control- MF-MA	0.000
	Control- Kooliner® control	0.000
	Control- Tokuyama® Rebase II control	0.000
	MMA- MF-MA	0.000
	MMA- Kooliner® MMA	0.000
	MMA- Tokuyama® Rebase II MMA	0.000
	MF-MA- Kooliner® MF-MA	0.000
	MF-MA- Tokuyama® Rebase II MF-MA	0.000
Kooliner®		
	Control- MMA	0.023
	Control- MF-MA	0.000
	Control- Tokuyama® Rebase II control	0.000
	MMA- MF-MA	0.005
	MMA- Tokuyama® Rebase II MMA	0.000
	MF-MA- Tokuyama® Rebase II MF-MA	0.000
Tokuyama® Rebase II		
	Control- MMA	0.008
	Control- MF-MA	0.000
	Control- Adhesive	0.000
	MMA- MF-MA	0.000
	MMA- Adhesive	0.978
	MF-MA- Adhesive	0.000

Discussion

The present study compared the bond strength of relined denture base using different surface treatments and hard reline materials as demonstrated by flexural strength. Vallittu *et al.,* 1994 concluded that MMA wetting time of 180 s was recommended to strengthen repaired acrylic resin.[18] In addition, Thunyakitpisal *et al.,* (2011) found that applying an MF-MA solution on the denture base surface for 15 s before repair significantly increased its flexural strength.[21] Thus, the present study used surface treatments with MMA 180 s and MF-MA for 15 s to improve the bond strength between the hard reline and denture base materials. Unifast Trad® and Kooliner® do not have adhesive from the manufacturer, thus this study did not apply adhesive in this group.

For each hard reline material, the mean flexural strength of various solvent treated groups were significantly higher compared with the untreated group. The bonding mechanism of relined denture base occurs when the surface treatment solvents dissolves and swells the denture base surface and evaporates, causing swelling of the surface layers. The monomer in the reline material subsequently diffuses and penetrates into the pores of the denture base and polymerizes to form an interpenetrating polymer network.[27] Three solvents were used for the denture base surface treatment (MF-MA, MMA, and Tokuyama Rebase II adhesive (ethyl acetate and acetone)). The dissolution efficiency can be explained by the relative closeness of solubility parameters and polarities of PMMA and the solvents. The solubility parameters of various solvents are closed to acrylic denture base (PMMA, 18.3 MPa½). These solubility parameters of MMA, MF, MA, ethyl acetate, and acetone are 18.0, 20.9, 19.6, 18.2 and 19.7 MPa½, respectively. The first null hypothesis was rejected.

For each hard reline material, the mean flexural strength of the MF-MA treated group was significantly higher than that of the MMA treated group and the manufacturer adhesive treated group (for Tokuyama® Rebase II Fast). The MF, MA and MMA have similar polarities due to their methyl ester groups that enhance their ability to soften an acrylic denture base while the other solvents have different functional groups. Acetone has ketone group. Ethyl acetate is ethyl ester. The dissimilar polarity of ethyl acetate and acetone to PMMA is likely to bring these compounds out of the range of effective solubility. In addition, the molecular weight of the solvent has an effect on the softening efficacy in which lower molecular weight promotes the faster kinetics of diffusion. MF (60.05 Da), MA (74.08 Da), acetone (58.08 Da), and ethyl acetate (88.11 Da) have a lower molecular weight than MMA (100.12 Da) which promotes greater solubility to the denture base.[26]

The boiling point of solvents also affects the bonding process that lower boiling point of solvent causes easier evaporation and takes less chair-time. Methyl formate (31.8°C) has the lowest boiling point compared to the other solvents. Methyl acetate (56.9°C) and acetone (57°C) have a similar boiling point. Ethyl acetate and MMA have a higher boiling point of 77.1°C, 101°C, respectively. A higher molecular weight and boiling point of MMA might provide lower solubility to the acrylic denture base material compared to the MF-MA solution. Ethyl acetate and acetone (in Tokuyama® Rebase II Fast adhesive) has a similar solubility parameter compared to PMMA but they have different functional groups in their chemical structure. Besides, ethyl acetate has a higher molecular weight and boiling point compared to MF-MA solution and acetone. Acetone has many requirements to promote PMMA dissolution similar to MF-MA except the different functional groups in chemical structure. The second null hypothesis was rejected.

In the same surface treatment, the flexural strength of Unifast Trad® relined group was significantly higher compared with those of the Kooliner® and Tokuyama® Rebase II Fast relined groups. The monomer (in liquid part) with a lower molecular weight can diffuse and penetrate

and form an interpenetrating polymer network better than that with high molecular weight. The Unifast Trad® liquid contains MMA (100.12 Da) that are lower in molecular weight compared with the IBMA (142.20 Da) in Kooliner, or AAEMA (214.21 Da) and 1,9 NDMA (296.40 Da) in Tokuyama® Rebase II Fast.[28] Thus, the third null hypothesis was also rejected.

Conclusion

Surface treatment with MF-MA solutions significantly increases the flexural strength of a relined denture base. This study suggests the application of MF-MA solutions for 15 s before the relining procedure to improve the flexural strength between the denture base and hard reline materials.

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Research Articles

Effects of Provisional Cements on Shear Bond Strength of Resin Cements to Dentin

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Abstract

This study evaluated the effects of provisional cements on the shear bond strength (SBS) of permanent resin cements to dentin. The buccal cusps of extracted human mandibular first premolars (n=144) were sectioned to expose dentin at 3 mm from the buccal cusp tip. The specimens were first equally divided into four groups according to the provisional cements used: control (no cementation), zinc oxide eugenol, zinc oxide non-eugenol and calcium hydroxide. The provisional cement was mixed and applied on the dentin surface with an acrylic rod placed over with 10 N constant load until the cement was set. The test specimens were stored in distilled water at 37°C for one week. The acrylic rods were removed and provisional cement remnants were cleaned with spoon excavator and pumice-water slurry. The specimens were then divided equally into three subgroups for testing permanent resin cements: self-adhesive, self-etch and total-etch. Permanent resin cement was used to cement a composite resin stick onto dentin surface. After 24 hours, all specimens were processed to test the shear bond strength with a universal testing machine. Data were analyzed with two-way ANOVA and Tukey's test. There was no interaction between provisional and permanent resin cement groups. The bond strength obtained when using calcium hydroxide and zinc oxide eugenol provisional cement were similar to no provisional cement contamination. Zinc oxide non-eugenol provisionalization had a significantly lower shear bond strength than the others. The shear bond strength of the total-etch cement group was the highest while that of the self-adhesive group was the lowest. In conclusion, the shear bond strength of three permanent resin cement is not affected when using zinc oxide eugenol and calcium hydroxide as provisional cements, but is reduced when using zinc oxide non-eugenol cement.

Keywords: Provisional cement, Resin cement, Shear bond strength (SBS)

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Introduction

During the processes of fabrication of the permanent fixed restoration, the provisional restorations with provisional cementation are required to maintain chewing function, pulpal protection and esthetic.[1] The provisional cement should be firm enough to retain the provisional restoration in place, while it should be easy to be removed and has no effect on permanent cementation.[2] Available in the market are calcium hydroxide liners such as Dycal® (DENTSPLY Caulk, Milford, DE, USA), zinc oxide cements such as Temp-Bond™ (Kerr, Orange, CA, USA) and zinc oxide non-eugenol cements such as Temp-Bond™ NE (Kerr, Orange, CA, USA).[2-4]

The reduction in the bond strength of permanent cementation has been found after the tooth surface is contaminated with provisional cements.[5-7] Eugenol (4-allyl 2-methoxy phenol) in provisional materials has been blamed to interfere with the polymerization process of resin materials, because of its antioxidizing property.[8] It might interfere with the retention of permanent resin cement.[1,9] As a result, the zinc oxide non-eugenol has been manufactured to serve this purpose.

Calcium hydroxide liners such as Dycal® has also been used as provisional cement with the advantage of higher mechanical and adhesive properties over zinc oxide cement.[10] However, in the moist condition, it releases hydroxyl ions that creates alkaline environment which initially irritates dental pulp, but later stimulates reparative dentin formation.[11] Its alkaline pH could also neutralize a mild acidic resin primer of the self-etch system and a mild acidity of the self-adhesive cement.[6,12]

The bonding ability of permanent cementation determines the success of fixed restoration.[13] Resin cements have been widely used for permanent cementation due to their high bond strength, low solubility and acceptable esthetic.[14] They have been classified into three systems based on adhesive mechanisms; total-etch, self-etch and self-adhesive. Total-etch system (such as Variolink® N, Ivoclar Vivadent, Schaan, Liechtenstein) treats dentin surface with 35-37 % phosphoric acid to create microporosities before applying primer and bonding agent to form hybrid layer and resin tag for micromechanical interlock.[15] Self-etch system (such as PANAVIA™ F2.0, Kurarey, Osaka, Japan) prepares tooth surface with acidic functional monomers, for example, 10-MDP (10-methacryloyloxydecyl dihydrogenphosphate) to enhance chemical bond to calcium ions in hydroxyapatite on tooth surfaces before applying bonding agent.[16,17] Self-adhesive system (such as Rely X™ U200, 3M ESPE, Seefeld, Germany) has been recently developed by combined all acidic, primer, and adhesive agents into a single mixture of cement for the simplest use with less chance of postoperative hypersensitivity.[18-20]

There are controversial results from the literatures, whether eugenol, or residual of provisional cement disturb the etching quality, impair infiltration of adhesive into dentin or inhibit the polymerization of resin cement causing the reduction in the bond strength.[1,3,21] The aim of this study was to verify the effect of three provisional cements (zinc oxide eugenol, zinc oxide non-eugenol and calcium hydroxide) and the control with no provisional cement contamination on the shear bond strength of three resin cements (total-etch, self-etch and self-adhesive) to dentin.

Materials and Methods

This study has been approved by The Human Experimentation Committee of the Faculty of Dentistry, Chiang Mai University, Thailand. (Certificate of ethical clearance No. 1/2019). Human non-carious permanent mandibular first premolar teeth (n=144) were extracted as part of the orthodontic treatment. The samples were cleaned and stored in 0.1 % thymol solution. The root was cut off at the level of 3 mm apically to the cemento-enamel junction. Dentin under the buccal cusp was exposed by sectioned off with slow-speed diamond saw sectioning machine (Isomet® 1000 precision saw, Buehler, U.S.A) under water coolant, at the level of 3 mm from the buccal cusp tip (Fig. 1). The tooth specimen was positioned and held with dental stone type IV in polyvinylchloride (PVC) tube with the exposed cut tooth surface upward.

Dentin surface of all specimens was polished with 600-grit silicon carbide paper with water for 10 seconds to create standard smear layer. The prepared dentin surfaces were evaluated under a stereomicroscope system and digital camera (SZX7 & SZ2-ILST led illuminator stand & E-330, Olympus, Tokyo, Japan) at 40x magnification to verify complete enamel removal, no pulpal exposure or crack.

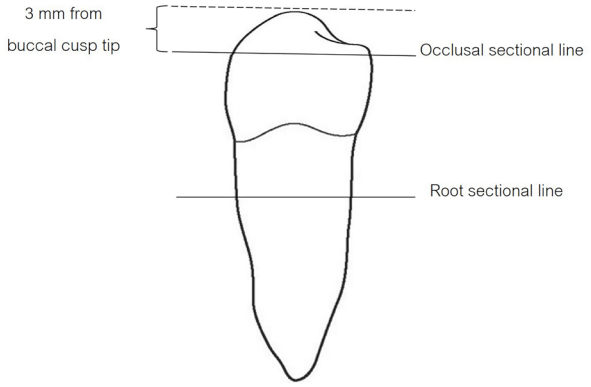

Figure 1 *Schematic diagram shown sectional lines for tooth specimen preparation.*

For testing the effect of provisional cements, the specimens were randomly divided equally into four main groups (n=36). One group served as a control while the other three groups were used for testing three provisional cements; calcium hydroxide liner (Dycal®), zinc oxide non-eugenol cement (Temp-Bond™ NE) and zinc oxide eugenol cement (Temp-Bond™). The provisional cement was applied onto the dentin surface before placing an acrylic rod (12 mm in diameter and 5 mm in height) over with 10 N constant load. The excess cement was gently removed using sharp tip explorer. After the cement had set, the specimen was kept in distilled water at 37°C.

After seven days, the acrylic rods were removed from the tooth surface. The cement remnants were removed using spoon excavator followed by polishing with slurry water of fine grain pumice using prophylaxis rubber cup with slow speed handpiece for 10 seconds and cleaned with water spray for 5 seconds. An adhesive tape (Paper Masking Tape No.720, Nitto Denko, Osaka, Japan) with 100 µm thickness and 3 mm hole was placed over the cut tooth surface for restricting the bonding area between permanent resin cement and dentin.

Resin rods (3 mm in diameter and 3 mm in height) were made from a light-cured composite resin (Filtek™ Z350 XT, 3M ESPE, Seefeld, Germany). Theirs bonding surface was prepared by air blasting with a 50 µm aluminum oxide under 35 PSI of pressure using airborne-particle abrasive unit (Basic Classic, Renfert GmbH, Hilzingen, Germany) and cleaned with ultrasonic cleaner machine (Easyclean, Renfert GmbH, Hilzingen, Germany).

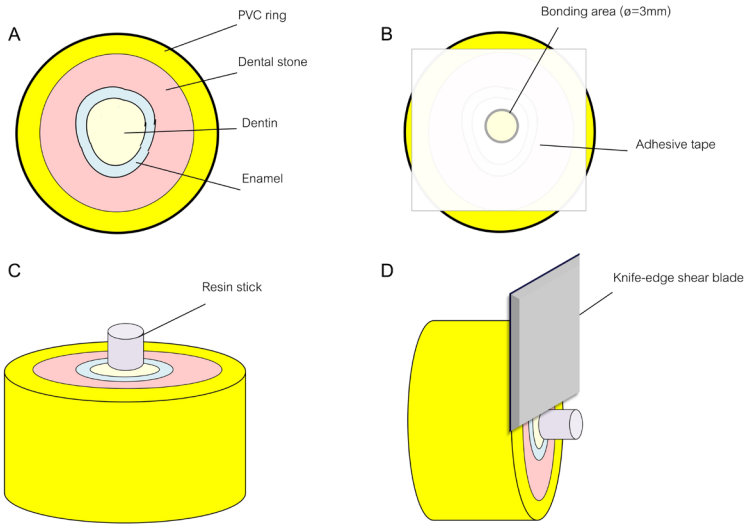

Figure 2 *(A) prepared tooth specimen was embedded in PVC ring (top view) (B) an adhesive tape with 3 mm hole was placed on prepared dentin surface for restricting the bonding area (top view) (C) resin rod was place on the tooth specimen with 10 N constant load (D) SBS test using knife-edge shear blade at resin-dentin interface.*

Each main group (n=36) was divided further into three subgroups (n= 12) for testing three permanent resin cements; total-etch, self-etch and self-adhesive. The tooth surface was prepared and three permanent resin cements were used according to the manufacturer's instruction. In brief, for self-adhesive cement (Rely X™ U200), the tooth surface was prepared by dropping distilled water on dentin with gentle air blow until the dentin surface was slightly shiny with moist, then resin base and catalyst were mixed. For self-etch cement (PANAVIA™ F2.0), self-etching primers (ED Primer II Liquid A and B) were mixed and applied on the moist bonding area with agitating technique for 15 seconds, waited for 15 seconds and air-dried until no movement of the liquid. Resin base and catalyst pastes were mixed for 20 seconds. For total-etch cement (Variolink® N), dentin surface was treated with 37 % phosphoric acid for 15 seconds, rinsed with water for 20 seconds, gently air blew until dentin surface appeared moist, applied Syntac primer and left it dried for 15 seconds, applied Syntac adhesive, dried after waiting for 10 seconds, applied Heliobond and blew to a thin layer, then mixed base and catalyst pastes of the resin cement.

The mixed resin cement was applied onto both prepared dentin surface and treated surface of resin rod. The resin rod was placed in the hole of adhesive tape onto dentin surface with 10 N constant load for controlling the cement thickness (according to ISO/TS 11405, 2015).[22] The curing light from a light-curing unit (Elipar™ LED Curing Light, 3M ESPE, Seefeld, Germany) with radiances of 1000-1200 mW/cm^2 was shone on the cement for 2 seconds then removed surrounding excess cement, and subsequently applied light for 20 seconds in four different directions of resin-dentin interface for complete polymerization.

The specimens were kept in distilled water at 37°C for one day before preparing for the SBS test in a universal testing machine (Instron® 5566, Instron Limited, Massachusetts, U.S.A) with 50 kg load cell. A knife-edge shear blade was positioned to compress at the cement interface of dentin and resin rod with a cross head speed of 0.5 mm/minute. The force was read out in newton (N) and was calculated to be the shear bond strength values in megapascals (MPa) by divided with the area of the bonding interface.

The failure mode of specimens was evaluated using a stereomicroscope at 50x magnification. The fracture surfaces were classified as adhesive failure (failure at dentin-resin interface), cohesive failure (within dentin, cement or resin rods) and mixed failure. Moreover, the six specimens of each group were randomly selected to be examined in more details under scanning electron microscope-SEM (JSM-5910LV, Jeol, Massachusetts, USA). The selected specimens were sectioned longitudinally. The cut surface was treated with 37 % phosphoric acid for 15 seconds to demineralize an inorganic part, followed by immersion in 5.25 % sodium hypochlorite for 20 minutes to remove an organic part and put in ultrasonic cleaner to remove all debris from the surface and examined under SEM.

Two-way ANOVA statistical analysis was used to investigate the interaction between provisional and permanent cements and Tukey's post hoc test for pair-wise comparisons (α = 0.05) to identify the difference within the same group.

Table 1 *Shear bond strength (SBS) was presented in mean ± standard deviation values for both provisional cement and permanent cement groups.*

Provisional cement (n=36)	Permanent cement (n=12)	SBS Mean±SD (MPa)	
None	Self -adhesive	6.41±1.06	** *
	Self-etch	7.94±1.23	
	Total-etch	8.71±1.14	
Calcium hydroxide	Self-adhesive	6.64±1.13	*
	Self-etch	7.38±1.03	
	Total-etch	8.42±1.20	
Zinc oxide eugenol	Self-adhesive	6.59±1.28	**
	Self-etch	7.47±0.78	
	Total-etch	8.16±1.59	
Zinc oxide-non eugenol	Self-adhesive	5.91±0.84	*
	Self-etch	6.17±1.15	**
	Total-etch	7.74±1.45	

**indicated the significant difference (p<0.001)*

***indicated the significant difference (p<0.05)*

Results

Two-way ANOVA statistical analysis suggested that there was no interaction between provisional and permanent cements ($p \geq 0.05$), but found a significant difference within the provisional cement main groups ($p<0.05$) and within the permanent cement main groups ($p<0.001$). The mean ± standard deviation of the shear bond strengths in MPa among control and the 3 provisional cement groups with the 3 permanent cement groups were presented in Table 1.

The multiple comparison using Tukey's test among subgroups of the cements suggested that zinc oxide non-eugenol group had a significant ($p<0.05$) lower shear bond strength (6.61±1.40 MPa) than that of the control (7.69±1.47 MPa), calcium hydroxide (7.48±1.32 MPa) and zinc oxide eugenol (7.41±1.39 MPa) (Fig. 3A) and there were significant differences among the three permanent resin cements, while the total- etch cement had the highest SBS (8.26±1.36 MPa) and the self-adhesive cement had the lowest SBS (6.39±1.09 MPa) (Fig. 3B).

For the self-etching cement, zinc oxide non-eugenol group had a significant lower bond strength (6.17±1.15 MPa) when compared to the control (7.94± 1.23 MPa) ($p<0.001$) and the zinc oxide eugenol group (7.47±0.78 MPa) ($p<0.05$) while other groups were not different. There was no significant difference when comparing among provisional cement groups for total-etch and self-adhesive cements.

For all permanent cement groups, total-etch cement and self-etch cement had only the mixed type of failure mode (100 %), while the adhesive type of failure mode when using self-adhesive were 58.33 %, 66.67 %, 66.67 % and 83.33 % for calcium hydroxide group, zinc oxide eugenol group, zinc oxide non-eugenol group and control group, respectively. From SEM images, long resin tags with lateral branches, with an average length of 20-50 µm, were found in the total-etch group. Self-etch group showed absent or short resin tags with the average length about 5-10 µm, while there was no resin tag in none of the specimen in the self-adhesive group.

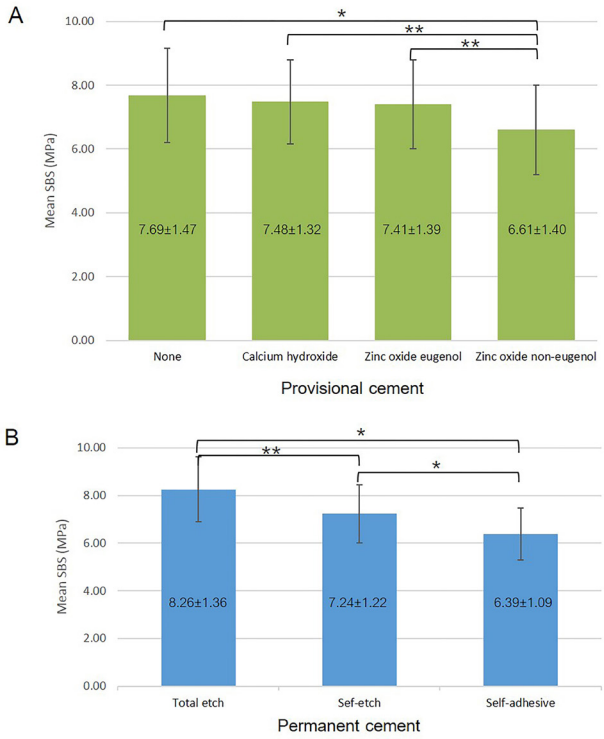

Figure 3 *Shear bond strength mean values ± standard deviation within cement groups (A) provisional cement groups (B) permanent cement groups. [*indicated the significant difference (p<0.001) **indicated the significant difference (p<0.05)]*

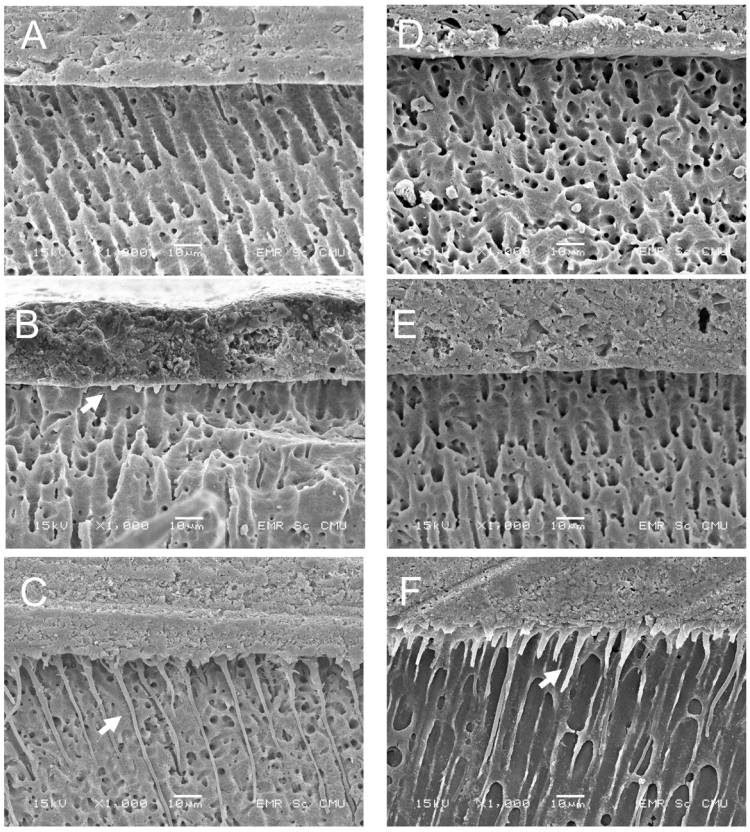

Figure 4 *SEM images of resin-dentin interface demonstrated resin tags (arrow) and dentinal tubule at the magnification of x1,000 (A-C) a sample in the control groups, (D-F) zinc oxide non-eugenol groups (A,D) samples were permanently cemented with self-adhesive cement, (B,E) self-etch cement, (C,F) total-etch cement. The SEM images of calcium hydroxide and zinc oxide eugenol showed similar results with these two groups.*

Discussion

Zinc oxide non-eugenol cement had a significant lower shear bond strength when compared to the control, calcium hydroxide cement and zinc oxide eugenol groups. This coincided with the observation by Altintas and colleagues[1] that zinc oxide non-eugenol had the lowest SBS compared to no provisional cement contamination and calcium hydroxide cement groups. Moreover, this supported the evidences that the use of eugenol-containing cement did not have an effect on bond strength of permanent resin cement,[23] but the use of zinc oxide non-eugenol did. In contrast to some studies[4,9] that showed the opposite result.

The bonding between resin cement and dentin could be affected by multiple factors. The duration of seven days for provisional restoration should be suitable for clinical practice because it is relevant to the duration for laboratory processes to make permanent fixed restoration. According to a systematic review by Ajaj and colleagues, eleven studies showed no significant adverse effect on the bond strength of permanent resin cementation when tooth surfaces were contaminated with eugenol-containing provisional materials for seven days,[24] while another study suggested that provisionalization with calcium hydroxide cement for a short-term as 7 or 30 days did not affect the bond strength of permanent adhesive resin to dentin.[25] The alkaline property of calcium hydroxide cement was the concern that it might neutralize the acidic capacities of adhesive systems or alter the organic matrix in the dentin.

On the contrary, other studies found a significantly reduced bond strength when provisionalization with eugenol-containing cement for one day.[24] Hume and colleagues[26] suggested that eugenol was released from hydrolysis of zinc oxide eugenol into dentin at the highest rate during the first day of placement and decreased thereafter. This supports the result of this study that the zinc oxide eugenol provisional cementation for 7 days has no effect on the bond strength of permanent resin cement.

The result of the bond strength of permanent resin cement, of this study, showed no significant difference among the control, calcium hydroxide and zinc oxide eugenol groups. This suggests that the surface of dentin of these two provisional cementation groups are not different from that of the control group. Moreover, the technique of using spoon excavator to remove cement remnants on the tooth surface followed by polishing with pumice-water slurry could provide acceptable bond strength and simple to use in the clinic.[24,27] However, the reduction in bond strength of zinc oxide non-eugenol has been unclear and could be caused by other factors.

Feitosa and colleagues[28] reported that the presence of Zn^{2+} in the form of zinc oxide might compromise the performance of MDP in forming MDP–Ca salts and reduce the bonding of MDP-based self-etch adhesives. The provisional cements used in this study contain different proportion of zinc oxide in the base. Under acidic condition, zinc oxide could dissociate from cement and bind to phosphoric functional monomer instead of calcium ion resulting in compromised bonding interface.

In moist condition, calcium hydroxide can be dissolved and release hydroxyl ions[29] which could disturb the acidic capacity of resin cement, deteriorate organic matrixes and reduce strength of dentin structures.[30, 31] The result of this study found the bond strength, when using calcium hydroxide as provisional cement, to be similar to that of the control which is in accordance with others studies.[5,32] It could be attributed to the high solubility in water of Dycal® (4.21 %) so this cement could be easily eliminated during the cleaning process.[33]

Similar to other studies,[32,34] total-etch cement has the highest bond strength, followed by self-etch and self-adhesive cements. The bond strength of self-etch cement was reduced significantly when using Temp-Bond™ NE as a provisional cement similar to the study by Carvalho and colleagues.[23] The total-etch system has the advantage of a higher bond strength than the others, but it also has disadvantage on several steps of application. Strong

phosphoric acid used in total-etch system is most effective in removing smear layer, smear plug and other inorganic materials allowing bonding agents to penetrate deep into the dentinal tubule and form long resin tags[34-36] as shown in Figure 4C, 4F. Self-etching system simplifies clinical procedure and reduces technique sensitive of the total-etch system. Primer has mild acidic property (pH >2) which is less effective on removing smear layer and smear plug. Short or absent resin tags were found in the hybrid layers in SEM images (Fig. 4B, 4E) which is in accordance with other investigations.[37,38] Self-adhesive cement (Rely X™ U200) has an advantage on clinical application, but it has low acidity (pH = 2.8)[39] resulting in the lowest potential to remove smear layer or cement debris and the lowest bond strength compared to other systems.[12,23,34] The SEM images (Fig. 4A, 4D) showed no resin tag penetration and the tubular orifices was covered with smear layers.

This study used extracted teeth which might have some limitations such as the lack of continuous outward flow of dentinal fluid, but a small area of the bonding area can be controlled to be comparable between specimens. Dentinal fluid in vital tooth could disturb hybrid layer formation and resin infiltration into the dentinal tubule to form resin tags. So, the long resin tags found in total-etch cement in this *in vitro* study could be shortened or absent when the dentinal fluid flow is present. Further studies should be conducted to evaluate the bond strength of permanent resin cement under simulated pulpal pressure or *in vivo*. Moreover, residual element particles of provisional cement remnants on the dentin surface should be investigated

Conclusion

Under the conditions used in this experiment, the shear bond strength of three permanent resin cements is not affected when using zinc oxide eugenol and calcium hydroxide as provisional cement, but is reduced when using zinc oxide non-eugenol cement. Among the three permanent cements, the total- etch adhesive cement yields the highest SBS, while the self-adhesive cement gives the lowest value.

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Original Article

Efficacy of Double Antibiotics in Hydroxypropyl Methylcellulose Gel against Enterococcus faecalis in Root Canals: An *in vitro* Study

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Abstract

Ciprofloxacin and metronidazole exert an antibacterial activity against Enterococcus faecalis *(E.f.).* However, applying paste containing these antibiotics into the root canals is not convenient for endodontists. This study aimed to study the possibility of hydroxypropyl methylcellulose (HPMC) as a vehicle for antibiotic delivery. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the two antibiotics were determined. A 1:1 ratio of each antibiotic was mixed with 2 % HPMC to form a gel, and the *in vitro* drug release was tested by a Franz diffusion cell. Fifty-four roots of the mandibular premolars were mechanically instrumented by Protaper Next rotary files until X3 and divided into three groups (18 each) (i) non-infected roots, treated with gel base, (ii) infected roots with *E.f.* for 21 days, treated with gel base, and (iii) infected roots with *E.f.* for 21 days, treated with gel containing the combined antibiotics for 14 or 28 days. DNA and RNA were isolated from the ground roots. The absolute quantity of *E.f.* DNA and relative mRNA expressions of *E.f.*-specific sequence and *pbp5* were determined by qPCR and RT-qPCR, respectively. MIC and MBC of the double antibiotic solution were 5 and 250 µg/ml, respectively. There was a significant decrease in the *E.f.* DNA content in group (iii) at 14 and 28 days ($p<0.001$). mRNA expression of *E.f.*-specific sequence was significantly reduced in group (iii) at both periods ($p<0.01$), whereas *pbp5* expression was significantly increased ($p<0.01$). This study demonstrated an *in vitro* efficacy of the combined antibiotics in the HPMC gel against *E.f.*, proposing an application for endodontic treatment.

Keywords: Biodegradable gel, Double antibiotics, Enterococcus faecalis, Intracanal medication, Root canal treatment

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Introduction

Infections with mixed bacteria play a major role in the development and progression of pulp and periapical diseases.[1,2] A major goal for root canal treatment is to eliminate bacterial populations. Controlled asepsis by means of mechanical preparation and chemical agents is important for successful healing of the periapical lesions.[3] Accordingly, intracanal medication with antibiotics is recommended to effectively remove residual bacteria after mechanical instrumentation.[4] The success rates of endodontic treatment for teeth without apical periodontitis range from 82.8 to 97.3 %, whereas those for teeth with apical periodontitis are lower varying from 75.6 to 87.77 %[5-7], as a result of persistent infections with gram-positive and facultative anaerobes.[8] Among these anaerobes, *Enterococcus faecalis* (*E. faecalis*) is the most prevalent bacteria found within previously treated canals.[9,10] *E. faecalis* is isolated alone or with a few other bacteria[11], and can penetrate and reside in the dentinal tubules, which helps resist killing by calcium hydroxide.[12] Moreover, a proton pump is critical for *E. faecalis* survival at high pH.[13] *E. faecalis* can enter the viable, but non-culturable (VBNC) state, in which it reduces metabolisms and synthesis of proteins, except penicillin binding protein 5 (*pbp5*), and remains dormant until necessary nutrients are available for its later growth.[14,15] Therefore, *pbp5* expression is used to determine *E. faecalis* in the VBNC state.[16]

Triple antibiotic paste (TAP), containing ciprofloxacin, minocycline and metronidazole in propylene glycol and macrogol, has been used as an intracanal medicament for selective root canal treatment and pulpal revascularization. Several studies have, however, shown that minocycline causes visible crown discoloration.[17] Therefore, minocycline is omitted from the paste, and called double antibiotic paste (DAP). *In vitro*, DAP was as effective as TAP against *E. faecalis* in the root canal[18,19], as evidenced by the resolution of periapical lesions by intracanal medication with DAP as reported by Iwaya *et al.*,[20] and Hargreaves *et al.*,[21] Nevertheless, mixing minocycline and ciprofloxacin with propylene glycol and macrogol as traditional vehicles makes the paste too viscous to be readily delivered or to fill up the root canal, limiting the penetration of antibiotics into the dentinal tubules. In addition, propylene glycol cannot control a sustained drug release.[22]

In this study, we wanted to determine the possibility of using hydroxypropyl methylcellulose (HPMC) as a vehicle for antibiotic delivery. HPMC, a water-soluble and biodegradable polymer derived from cellulose, is the most abundant polymer in nature and is used in food, drug and dietary supplements.[23] A previous study revealed a sustained release of drugs by using HPMC as a vehicle, which effectively prolongs their therapeutic effect.[24] To the best of our knowledge, HPMC has not yet been introduced for dental uses and no study has so far investigated the antibacterial activity of ciprofloxacin and metronidazole in HPMC gel. Therefore, this study aimed to examine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the two antibiotics against *E. faecalis in vitro*, to evaluate the release of these antibiotics from the gel, and to assess the antibacterial efficacy of the combined antibiotics in the gel against *E. faecalis* at 14 and 28 days.

Materials and Methods

E. faecalis Strain and Medium

Blood agar plates (Merck KGaA, Darmstadt, Germany) were used to grow and maintain the colonies of *E. faecalis* ATCC 29212. A single colony was picked and inoculated in Brain-heart infusion (BHI) broth (HiMedia Laboratories Pvt. Ltd., Mumbai, India) supplemented with 5 g/L of yeast extract (HiMedia Laboratories Pvt. Ltd.) at 37°C for 24 h in an anaerobic chamber (Bactron, Shel lab, Lonay, Switzerland) for subsequent experiments.

Minimum Inhibitory and Bactericidal Concentrations

The double antibiotic solution was prepared by mixing 50 mg of ciprofloxacin and 50 mg of metronidazole (USP), generously obtained from the Siam Pharmaceutical Co., Ltd., Bangkok, Thailand, in 10 ml of deionized water, as recommended by Sabrah *et al.*,[19] The solution was serially diluted, ranging from 1:10 to 1:32000. *E. faecalis* cultures in BHI broth were treated with these dilutions in triplicate for 24 h in sterile 96-well microtiter plates

(Thermo Fisher Scientific, Waltham, MA, USA). The turbidity of *E. faecalis* cultures was determined by an optical density (OD) at 540 nm using a spectrophotometer (Tecan Austria GmbH, Grödig, Austria). MIC was determined as the fifty percent of growth inhibition[25] by the following equation: % of inhibition = (OD control - OD blank) - (OD sample - OD blank) x 100/(OD control - OD blank), where control = *E. faecalis* in BHI broth, blank = BHI broth alone, sample = *E. faecalis* in BHI broth treated with the solution. To determine MBC, bacterial cultures from the wells that contained different aforementioned dilutions were streaked onto blood agar plates, incubated at 37°C for 24 h in an anaerobic chamber. The lowest concentration of ciprofloxacin and metronidazole that resulted in no visible bacterial colony on the plates was considered as MBC.

In vitro Release of Double Antibiotics

An equal amount of ciprofloxacin and metronidazole (1:1) was dissolved in deionized water for the final concentrations of the combined antibiotics at 5 and 10 mg/ml. Two hundred mg of the HPMC powder (2 % w/v; S. Tong Chemicals Co., Nonthaburi, Thailand) was added to 10 ml of the double antibiotic solution and left at 4°C overnight to form the gel-like mixture. The *in vitro* release of both antibiotics from the mixture was performed using a Franz diffusion cell (Perme Gear, Inc., Hellertown, PA, USA) and artificial tubular cellulose membranes with a 0.4-µm pore size (Membrane Filtration Products, Inc., Sequin, TX, USA). Before being used, the membranes were hydrated with deionized water, as a medium, overnight. Two gram of the gel was loaded in the donor compartment, and 0.2 ml of the medium was withdrawn from the receptor compartment at various time points, including 0.25, 0.5, 1, 2, 4, 8, 12, 18 and 24 h. An equal volume of the fresh medium (deionized water) was immediately added into the receptor compartment at each sampling time to maintain sink condition. The samples were analyzed for the drug content using a spectrophotometer (UV-2450, Shimadzu Corporation, Kyoto, Japan).The standard curve of five known concentrations for each antibiotic was first established, and the concentrations of each antibiotic in the unknown samples were computed by comparing their OD values using the spectrophotometer with those of the five known concentrations from the standard curve. The OD readings, thus, indicated the accumulated concentrations of antibiotics at various times because some, but not all, medium was taken from the receptor compartment.

Preparation of Human Dentin Specimens

Fifty-four intact single-rooted premolars extracted for an orthodontic reason were collected, rinsed with normal saline and stored in 0.1 % (w/v) Thymol solution. The research protocol (#18/2018) of using discarded human premolars was approved by the Human Experimentation Committee, Faculty of Dentistry, Chiang Mai University. The root of each tooth was resected horizontally below the cementoenamel junction until the length of each root was equal to 10 mm. The equivalent size of each root canal was determined by using a K-file #15 (Dentsply Maillefer, Ballaigues, Switzerland), being fitted in the root canal at the apical one-third with the working length at 9 mm. The root canal was prepared by ProTaper Next rotary files from X1 to X3 (Dentsply Maillefer) following the manufacturer's instruction. The smear layer was removed by treatment with 17 % EDTA and 5.25 % NaOCl for 4 min each in an ultrasonic bath (UC-1050, TPC Advanced Technology, City of Industry, CA, USA). To test the sterility, all root samples immersed in BHI broth were autoclaved for 15 min, and a few samples were randomly selected and incubated in fresh BHI broth at 37°C for 24 h. No microbial contamination was found.

Dentin Inoculation

A single colony of *E. faecalis* grown on a blood agar plate was picked and suspended in BHI broth for 3 h. The quantity of *E. faecalis* in BHI broth was determined from a 0.5-OD value of the McFarland standard that is equivalent to 0.5 x 10^8 cfu per ml.[26] Thirty six of 54 root specimens were inoculated with *E. faecalis* at 1x10^6 cfu per ml by injecting 20 µl of *E. faecalis* suspension into the root canal close to the root apex using a 3-ml syringe and a 27-gauge needle. After inoculation, the root samples were incubated in an anaerobic chamber, and 20 µl of fresh BHI broth was replenished every two days for 21 days.

Subsequently, all root samples were assigned into three groups (n=18 each) as follows: (i) non-infected roots with 2 % HPMC gel (negative control); (ii) *E. faecalis*-infected roots with 2 % HPMC gel (positive control); (iii) *E. faecalis*-infected roots with 5 mg/ml of ciprofloxacin and metronidazole in 2 % HPMC gel (experimental group). A syringe tip was used to fill up the root canal with the HPMC gel with or without the two antibiotics by inserting into the canal around 7 mm away from the root apex (~2-3 mm) in order to prevent leakage of the gel outside the canal. In addition, slow loading of the gel was conducted to prevent any air trapped between the gel and the canal. The root samples held in 1.5-ml Eppendorf tubes were incubated at 37°C in an anaerobic chamber for 14 or 28 days (27 for each period with nine for each of the three aforementioned groups). The reason for intracanal medication with double antibiotics for 14 or 28 days was because we wanted to simulate a long period of medication, especially for the case of pulp necrosis with chronic abscess, and some patients might not be convenient to come back for a 7-day follow-up visit. After incubation, the root samples were irrigated with 10 ml of sterile normal saline followed by being dried with sterile paper points. Ten of the 18 root samples in each group (total = 30) were used for determination of the antimicrobial efficacy of ciprofloxacin and metronidazole in the HPMC gel by absolute quantification of *E. faecalis* DNA and mRNA expression of *E. faecalis*-specific sequence, whereas the remaining eight samples in each group (total = 24) were used for scanning electron microscopy.

DNA and RNA Isolation

The root samples were ground into fine powder by autoclavable steel impactors, driven by dual electromagnets, and vials (6751 vial, SPEX CertiPrep Ltd., London, UK) using a bone mill (SPEX SamplePrep Freezer/Mills 6750, SPEX CertiPrep Ltd.). The powder was collected and weighed in a 15-ml tube for DNA and RNA extraction. DNA and total RNA were simultaneously isolated using 1 ml of TRIzol™ reagent (Thermo Fisher Scientific) per 100 mg of the powder. Zero point two ml of chloroform per 1 ml of TRIzol™ reagent was added, vortexed and incubated for 3 min. The mixture was centrifuged at 12000 g for 15 min at 4°C. The aqueous phase in the upper layer was aspirated and precipitated with 0.5 ml of isopropanol for RNA pellet, while the interphase was collected and precipitated with 0.3 ml of absolute ethanol for DNA pellet. Both RNA and DNA pellets, resulting from centrifugation at 12000 g for 10 min and at 2000 g for 5 min, respectively, were washed with 75 % ethanol twice, left to air dry, and resuspended in 25 µl of RNase- and DNase-free water (Thermo Fisher Scientific). DNA and total RNA amounts were determined by a NanoDrop™ spectrophotometer (Thermo Fisher Scientific) and then stored at -80°C until further analysis.

Reverse Transcription and qPCR

Complementary DNA (cDNA) was synthesized from total RNA using the RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific). The reaction mixture contained 450 ng of total RNA, 1 µl of random hexamer primers and nuclease-free water up to 12 µl, 4 µl of 5x buffer reaction, 1 µl of Ribolock RNase inhibitor, 2 µl of 10 mM dNTP mix, and 1 µl of reverse transcriptase. The mixture was gently mixed and incubated at 25°C for 5 min followed by 42°C for 60 min. The reaction was terminated by heating at 70°C for 5 min. Two microliters of the resulting cDNA template were used for qPCR reaction in a total volume of 20 µl, containing 10 µl of 2x SensiFAST SYBR® No-ROX mix (Bioline, London, UK), 0.8 µl of 10 µM forward and reverse primers for *E. faecalis*-specific sequence, *pbp5* and *16s rRNA,* as a housekeeping gene (Table 1), and nuclease-free water. The reaction was performed in the LightCycler® 480 instrument (Roche, Basel, Switzerland) at 95°C for 10 min, followed by 45 cycles at 95°C for 20 s, 60°C for 1 min, and 72°C for 25 s. The cycle threshold (Ct) values of *E. faecalis*-specific sequence and *pbp5* were calculated using the LightCycler® 480 Software (Roche) and normalized by those of *16s rRNA* to obtain ΔC_t.

Table 1 *Oligonucleotide primers used in this study.*

		Oligonucleotide sequence
pbp5	Forward	5'-GATGCGCAATTAATCGG-3'
	Reverse	5'-CATAGCCTGTCGCAAAAC-3'
E. faecalis-specific	Forward	5'-CGCTTCTTTCCTCCCGAGT-3'
	Reverse	5'-GCCATGCGGCATAAACTG-3'
16s rRNA	Forward	5'-GATTAGATACCCTGGTAGTCCAC-3'
	Reverse	5'-TACCTTGTTACGACTT-3'

For absolute DNA quantification, 17 ng of DNA from each sample and of genomic DNA extracted from known concentrations (10^2 to 10^9 cfus/ml) of *E. faecalis,* for construction of a standard curve, were amplified in the qPCR reaction, containing 10 µl of 2x SensiFAST SYBR® No-ROX mix (Bioline), 0.8 µl of forward and reverse primers for *16s rRNA* (Table 1) and nuclease-free water. The qPCR conditions were 95°C for 10 min, followed by 45 cycles at 95°C for 20 s, 60°C for 1 min, and 72°C for 25 s. The quantity of *E. faecalis* DNA was derived by comparing the Ct value of each sample with the standard curve. Log concentrations of *E. faecalis* were plotted on a y-axis of the standard curve and Ct values of genomic DNA from *E. faecalis* were plotted on an x-axis.

Scanning Electron Microscope (SEM)

The remaining samples were split longitudinally with a chisel and a hammer into two pieces in order to view *E. faecalis* within the root canal and dentinal tubules. The root samples were fixed with 2.5 % glutaraldehyde in phosphate-buffered saline, dehydrated in serial dilutions of ethanol, dried with Polaron CPD7501 (Quorum Technologies Ltd., East Sussex, UK) and coated with gold using the gold-sputter-coated instrument (JFE-110E, JEOL, Tokyo, Japan). The coated samples were mounted on the stub and examined by an SEM (JSM-5410L, JEOL) at the magnification power of 7500x. The images were taken by a built-in digital camera attached to the SEM.

Statistical Analysis

Numerical data were checked for their distribution by the Kolmogorov-Smirnov test. Due to their normal distribution, the data were illustrated as mean percentages ± SD, representing log concentrations of *E. faecalis* DNA, and as means ± SD for mRNA expressions of *E. faecalis*-specific sequence and *pbp5* relative to that of *16s rRNA.* ANOVA and the Tukey's post-hoc tests were used to compare means with the significance level at *P*-values <0.05. Statistical analysis was performed using SPSS® software version 20 (IBM®, Armonk, NY, USA).

Results

MIC and MBC of the double antibiotic solution against *E. faecalis* were 5 µg/ml (2.5 µg/ml each; Table 2) and 250 µg/ml (125 µg/ml each; Fig. 1), respectively.

The maximum absorption of ciprofloxacin and metronidazole in deionized water was at 274 and 320 nm, respectively (data not shown), allowing simultaneous analysis of the antibiotics released from HPMC gel. Regarding the antibiotic release, 5 mg/ml and 10 mg/ml of the combined antibiotics in the HPMC gel were prepared according to Jenks *et al.,*[27] A sustained release of both antibiotics at these two doses was found up to 24 h (Table 3). Note that the concentrations of both antibiotics were close to the MIC and MBC at the same

time point, *i.e.* 15 min for the MIC and 2 h for the MBC, for both concentrations (Table 3). The concentrations of antibiotics released from 10 mg/ml of the combined antibiotics in the gel were higher than those from 5 mg/ml for every time point (Table 3).

However, the lower dose at 5 mg/ml of ciprofloxacin and metronidazole (2.5 mg/ml each) in the HPMC gel was selected for subsequent experiments because both 5 and 10 mg/ml yielded the MIC and MBC at the same time point and it is better to use the lowest dose of antibiotics that is still efficient in killing *E. faecalis* to avoid antibiotic resistance.[28] *E. faecalis* DNA amounts in the experimental group (iii) at 14 and 28 days were significantly decreased compared to those in the positive control group (ii) (p<0.001; Fig. 2A), but were not different from those in the negative control group (i) (Fig. 2A). mRNA expression of *E. faecalis*-specific sequence was significantly reduced in group (iii) at both periods compared to that in group (ii) (p<0.01; Fig. 2B). By contrast, mRNA expression of *pbp5* was significantly increased in group (iii) at both periods (p<0.01; Fig. 2C).

After dentin inoculation for 21 days, *E. faecalis* co-aggregated within the root canal and inside the dentinal tubules (positive control; Fig. 3). However, *E. faecalis* was dramatically eliminated by treatment with 5 mg/ml of ciprofloxacin and metronidazole in the HPMC gel with a few *E. faecalis* remaining in the dentinal tubules at 14 days (arrowheads; Fig. 3) and *E. faecalis* cell debris seen in the dentinal tubules at 28 days (arrows; Fig. 3). As anticipated, no *E. faecalis* was found inside the dentinal tubules in the negative control group (Fig. 3).

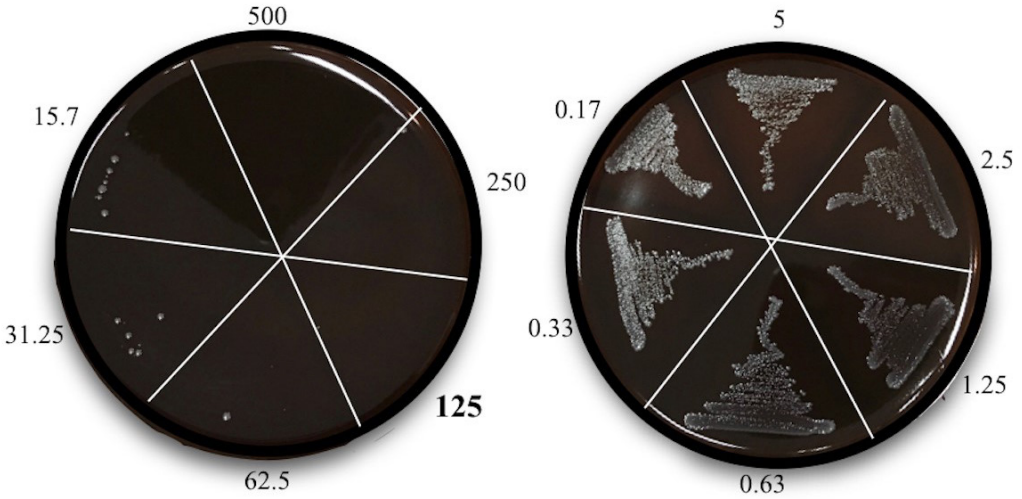

Figure 1 *Representative blood agar plates from three separate MIC experiments in Table 2 with similar results showing the minimum bactericidal concentration, in which no visible colony of E. faecalis was found at 125 µg/ml of each antibiotic (ciprofloxacin or metronidazole). Note E. faecalis cultures with twelve different concentrations of antibiotics in µg/ml from Table 2 were streaked onto different areas of two blood agar plates (six each).*

Table 2 *The percentage of E. faecalis inhibition by the double antibiotic solution, containing ciprofloxacin and metronidazole, in each dilution. Note that the fifty percent of inhibition regarded as the minimum inhibitory concentration was demonstrated at the dilution factor of 1:2000 or 5 μg/ml of the double antibiotic solution (bold). This experiment was done in triplicate on 3 separate times.*

Dilution	Double antibiotic (μg/ml)	Each antibiotic (μg/ml)	% of inhibition (mean ± SD)
1:10	1000	500	98.61 ± 0.91
1:20	500	250	96.35 ± 3.07
1:40	250	125	95.00 ± 3.93
1:80	125	62.5	89.84 ± 2.41
1:160	62.5	31.25	88.87 ± 1.80
1:320	31.25	15.7	86.64 ± 0.87
1:1000	10	5	76.00 ± 6.85
1:2000	**5**	**2.5**	**58.33 ± 4.40**
1:4000	2.5	1.25	44.94 ± 4.13
1:8000	1.25	0.63	34.26 ± 2.85
1:16000	0.63	0.33	23.34 ± 5.89
1:32000	0.33	0.17	13.86 ± 5.62

Table 3 *A profile of the sustained drug release for ciprofloxacin and metronidazole from 0.25 to 24 h, expressed as means ± SD in μg per ml. Note approximately equivalent doses of both antibiotics released at each time point for either 5 or 10 mg/ml of the combined antibiotics in hydroxypropyl methylcellulose (HPMC) gel. The accumulated concentration of each antibi otic released from either 5 or 10 mg/ml of the combined antibiotics in the gel reached the minimum inhibitory concentration at 2.5 μg/ml (light gray highlight) shown in Table 2 at 0.25 h and the minimum bactericidal concentration at 125 μg/ml (dark gray highlight) shown in Figure 1 at 2 h. This experiment was independently repeated three times.*

Time (h)	5 mg/ml of combined antibiotics in HPMC gel		10 mg/ml of combined antibiotics in HPMC gel	
	Ciprofloxacin (μg/ml)	Metronidazole (μg/ml)	Ciprofloxacin (μg/ml)	Ciprofloxacin (μg/ml)
0.25	3.03 ± 0.48	3.03 ± 0.40	3.62 ± 0.30	3.90 ± 0.59
0.5	32.61 ± 2.62	33.22 ± 2.66	43.28 ± 2.34	41.28 ± 3.63
1	6.91 ± 0.57	76.22 ± 1.27	88.56 ± 15.32	87.55 ± 2.93
2	144.33 ± 1.73	154.89 ± 10.51	194.55 ± 8.77	208.01 ± 5.53
4	259.79 ± 5.77	229.63 ± 14.20	345.66 ± 29.43	338.49 ± 7.06
8	410.45 ± 8.02	331.64 ± 16.49	513.72 ± 43.28	590.64 ± 3.25
12	440.62 ± 13.72	392.02 ± 7.40	623.72 ± 12.49	609.83 ± 8.52
18	449.22 ± 9.08	405.14 ± 5.45	649.99 ± 8.18	611.83 ± 7.14
24	457.23 ± 5.74	410.04 ± 5.98	664.24 ± 7.42	620.00 ± 4.29

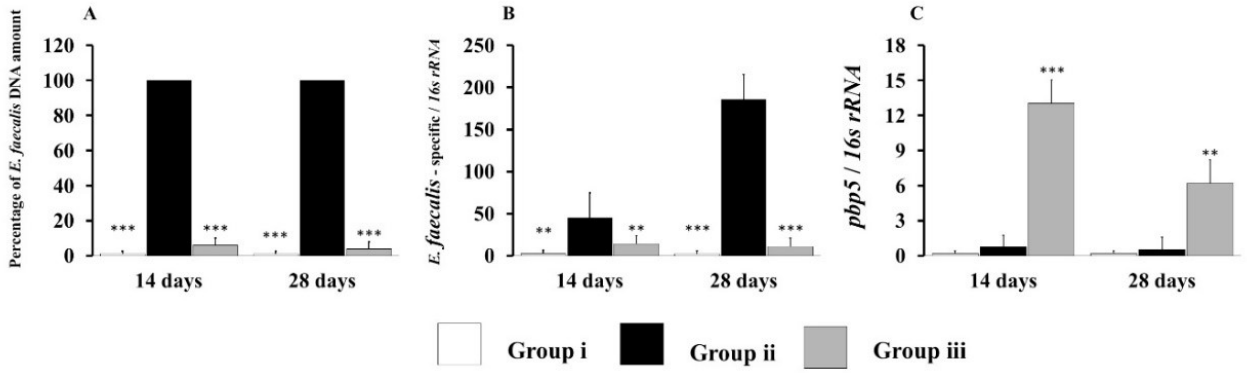

Figure 2 *Bar graphs demonstrating means ± SD (error bars) of the percentage of E. faecalis DNA (A), mRNA expression of E. faecalis-specific sequence normalized by that of 16s rRNA (E. faecalis-specific/16s rRNA; B), mRNA expression of pbp5 normalized by that of 16s rRNA (pbp5/16s rRNA; C) from five root samples in each group (n=5). ** = p<0.01; *** = p<0.001 for comparisons between group i or iii and group ii.*

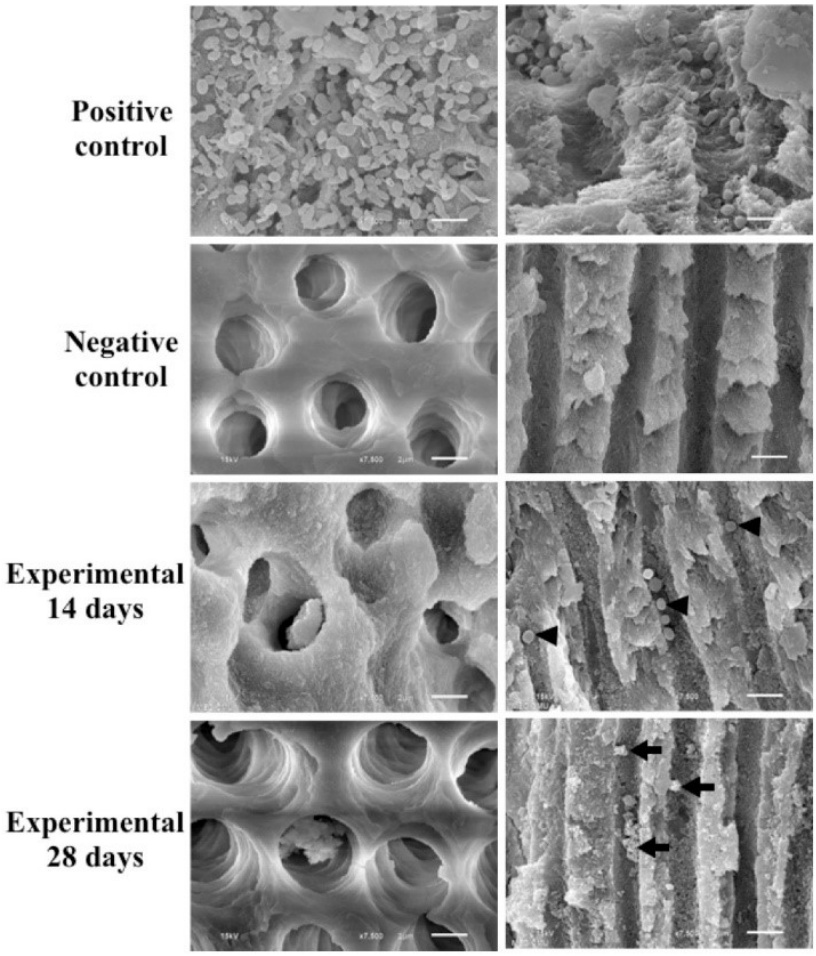

Figure 3 *Representative scanning electron micrographs from four different root samples in each group (n=4) with similar results showing almost eradication of E. faecalis by 5 mg/ml of ciprofloxacin and metronidazole in hydroxypropyl methylcellulose gel, at 14 and 28 days of treatment (experimental group). Arrowheads at 14 days showed some remaining E. faecalis in the dentinal tubules; arrows at 28 days indicated cell debris. The positive control was a root sample inoculated with E. faecalis for 21 days, while the negative control was a root sample without inoculation. Images on the left column were a cross-sectional view of dentinal tubules, while those on the right were a longitudinal view of the tubules. Bars = 2 µm.*

Discussion

In this study, MIC and MBC of the double antibiotic solution were 5 µg/ml (2.5 µg/ml each) and 250 µg/ml (125 µg/ml each), respectively. This MIC value is slightly higher than 1.4 µg per ml reported in previous study[18], due to different definition of MIC. The definition of MIC in this study was the fifty percent of *E. faecalis* inhibition, that was set as the lowest dose of ciprofloxacin and metronidazole that yielded a turbidity change ≤0.05.[18] However, the MBC value is approximately close to the MBC reported in previous study.[18] When ciprofloxacin and metronidazole were mixed with 2 % HPMC gel, a release of these antibiotics reached the MIC and MBC within 15 min and 2 h, indicating an immediate bacteriostatic action, followed by a bactericidal effect of the combined antibiotics in the HPMC gel. Moreover, a sustained release of both antibiotics up to 24 h was shown in Table 2. It is expected that these antibiotics can be released from the HPMC gel for a longer period of time than 24 h or until the antibiotics are completely released from the gel. Ciprofloxacin is a broad-spectrum antibiotic, whose structure is classified in the group of carboxy-fluoroquinoline. Its bactericidal action is due to the inhibition of two important enzymes, topoisomerase II (DNA gyrase) and topoisomerase IV, which are required for bacterial DNA replication, transcription, repair, strand supercoiling repair, and recombination.[29] Metronidazole is cytotoxic to facultative anaerobic bacteria by disrupting their energy metabolism and hindering their DNA replication, transcription and repair process, resulting in bacterial cell death.[30]

Regarding the *in vitro* bactericidal effect of ciprofloxacin and metronidazole in the HPMC gel, the DNA content of *E. faecalis* inoculated in the root dentin was remarkably reduced by treatment with the combined antibiotics in the HPMC gel for 14 and 28 days, consistent with a significant decrease in mRNA expression of *E. faecalis*-specific sequence and a considerable reduction of *E. faecalis* within the dentinal tubules (Fig. 3). Nevertheless, mRNA expression of *pbp5* was significantly up-regulated by the treatment with these antibiotics in the HPMC gel for 14 and 28 days, probably owing to a response of *E. faecalis* to environmental stress from antibiotic treatment or a transition of *E. faecalis* to the VBNC state.[31] Although the *pbp5* mRNA expression was significantly increased in group iii (hydrogel + double antibiotics) as compared to that in group ii (only hydrogel; Fig. 2C), the total number of their viable cells was considerably decreased by the double antibiotic treatment as shown by a significant and dramatic decrease in the mRNA expression of *E. faecalis*-specific gene (Fig. 2B). A very small amount of viable *E. faecalis* may not survive in the root canal or be sufficient to cause subsequent infection after proper sealing by root canal obturation. The *pbp5* expression in the experimental samples that contained the two antibiotics in the HPMC gel at 28 days was lower than that at 14 days (Fig. 2C). This may be due to a lower number of viable *E. faecalis* left in the root samples after prolonged antibiotic treatment. Conversely, the expression of *E. faecalis*-specific sequence at 28 days in the positive control that contained no antibiotics in the HPMC gel was higher than that at 14 days (Fig. 2B), possibly due to the growth of *E. faecalis*.

The HPMC gel has so far attracted considerable attention from both scientists and academicians in the biomedical field because of its excellent biocompatibility to human tissues and cells and low toxicity.[32] The efficacy of ciprofloxacin and metronidazole in the HPMC gel at 5 mg/ml against *E. faecalis* in this study is comparable to that of these antibiotics in methyl cellulose gel at 5 mg/ml[28], which effectively eliminates the bacterial biofilm in necrotic pulps from mature and immature roots.[33] However, there are some differences in the physical properties between HPMC and methyl cellulose. The solubility of HPMC in cold water is higher than that of methyl cellulose, so the HPMC can prevent droplets and particles from agglomerating, thus inhibiting the

formation of sediments.[34] Moreover, the viscosity of HPMC is less affected by a temperature change than that of methyl cellulose, and the HPMC gel is more stable to salts and to a wide pH range than the methyl cellulose gel.[24] Therefore, it is of our interest to develop the HPMC gel as a new vehicle for antibiotic delivery in the root canal due to these advantages of HPMC over methyl cellulose. Moreover, the efficacy of the double antibiotics in the HPMC gel was not compared with that of calcium hydroxide in the present study because few previous studies have demonstrated that calcium hydroxide does not exert an antibacterial activity against *E. faecalis.*[13, 18]

PCR, especially qPCR, is recommended for microbial detection and quantification because it is more sensitive than the conventional bacterial culture.[16,35] Furthermore, the PCR technique is suitable for detecting *E. faecalis* that enters the VBNC state, in which *E. faecalis* does not form a colony as it is cultured. *E. faecalis* in this state is still alive and can produce some enzymes and acids necessary for maintaining its pathogenicity[36], resulting in persistent root canal infections that lead to endodontic treatment failure. Consequently, the culture method would underestimate the total quantity of *E. faecalis* in the root dentin. A bone mill and steel impactors were used to grind the whole root into fine powder for simultaneous DNA and RNA extractions. Due to the presence of *E. faecalis* in the dentinal tubules (Fig. 3), pulverizing the whole root using these tools would yield more reliable DNA and RNA contents of *E. faecalis* than scraping the root dentin surface.[37]

A period of dentin inoculation with *E. faecalis* for 21 days was chosen to mimic chronic root canal infection and to show the existence of *E. faecalis* in the dentinal tubules.[38] This was verified by an SEM in the positive control (Fig. 3). Although 5 mg/ml of ciprofloxacin and metronidazole in the HPMC gel was effective against *E. faecalis in vitro* as early as 14 days with a further reduction of *E. faecalis* quantities at 28 days, it is still necessary to further determine the real efficacy of this preparation for treatment of pulp and periapical diseases in clinical settings. A long-term intracanal medicament up to 28 days is suggested in this study to maximally eradicate *E. faecalis* in the root dentin; however, a clinician's decision for root canal obturation is not made by *E. faecalis* quantities, but by clinical signs and symptoms.

Conclusion

In summary, 5 mg/ml of ciprofloxacin and metronidazole in the HPMC gel can significantly decrease *E. faecalis* inoculated in the root dentin, and their bactericidal effect lasts for at least 28 days. The HPMC gel may be clinically beneficial as a new vehicle for delivery of the double antibiotics into the root canal as an injectable, easy-to-flow and ready-to-use preparation of antibiotics mixed in the gel.

Conflict of Interest

The authors deny any conflicts of interest related to this study.

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Original Article

Association of Mixing Ability with Oral Status among Thai Elderly

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Abstract

The association of mixing ability with oral status is still limited in Thailand. This study aimed to investigate whether the mixing ability of older participant was associated with oral status, including the varying degree of occlusal support, periodontal status, and salivary flow rate. The cross-sectional study was conducted with 120 independently living older people aged 60 and over in Khon Kaen, Thailand. Dentate participants without denture replacement were recruited. Mixing ability was firstly measured by a two-color chewing gum mixing ability test with 20 chewing cycles and secondly determined the variance of hue (VOH) with the ViewGum software; inadequate mixing presents with a larger VOH. A calibrated dentist recorded the oral status and class of occlusal support according to the Eichner index. A structured questionnaire was used to obtain information on demographics, medical history, and oral health behaviors. Multiple linear regression analysis was used to evaluate the association between mixing ability and occlusal support while controlling for possible confounders. The result indicated that mixing ability was associated with occlusal support according to the Eichner index classification. (Beta: 1.041, 95% CI: 1.022 – 1.06), percentage of CAL ≥ 5 (Beta: 1.003, 95% CI, 1.002 – 1.004) but was not related to salivary flow rate after controlling for all possible confounders. This study suggested that mixing ability was associated with occlusal support according to Eichner index classification and periodontitis in the elderly.

Keywords: Eichner index, masticatory performance, periodontitis, salivary flow rate

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Introduction

Thailand is experiencing a demographic transition due to the aging population. By 2040, Thailand's population of older people is expected to increase to 25 % of the population.[1] Deleteriousness of the physical in the elderly leads to a variety of health problems, including oral health problems. Moreover, a national oral health survey revealed high levels of tooth loss and a high prevalence of periodontal disease, indicating poor oral health among Thai older people.[2] The World Dental Federation Organization (FDI World Dental Federation) has redefined the word "oral health" as an important part of general health both physically and mentally. Chewing and swallowing are included in the ability of oral function[3], which may be different from other aging changes because of a remarkable change in tooth loss.

The goal of the masticatory function is to break food down into discrete portions by chewing and mixing the aliment with saliva in order to form a bolus that is safe to swallow.[4] Besides these functional aspects; mastication plays an important role which is considered the first link in the chain for proper digestion and absorption of nutrients. It is reported that the number of cycles needed to chew a standard piece of food increases progressively with age, with increased particle size reduction and longer chewing sequence duration.[5] Several factors might compromise the masticatory function. Most commonly, the lack of teeth or saliva, as well as reduced muscular forces which seem to accelerate dysfunction with aging, are associated with an impaired chewing function.[6] Two-colored chewing gum[7] has been used to asses the degree of bolus formation and color-mixing and to quantify masticatory performance. The mixing ability test with the two-colored chewing gum is a good method to determine masticatory function in participants with a compromised masticatory performance.[8]

Therefore, it is necessary to study factors that may be linked masticatory function to the aging process. However, there is no epidemiologic study of the relationship between oral status and masticatory performance among Thai older people. Therefore, we conducted a study that aimed to investigate the relationship between oral status and mastication performance characterized by mixing ability of older people in Khon Kaen, Thailand.

Materials and Methods

This cross-sectional study was carried out among older people in the Muang District of Khon Kaen province during April-August 2017. The study protocol was approved by Khon Kaen University Ethics Committee for Human Research (HE602048). Eligibility criteria of participants included being 60 years of age or older at the time of the interview, and the ability to communicate. Exclusion criteria were: (1) having dementia or schizophrenia; (2) having a visual impairment, hearing impairment, or deafness; and (3) complete edentulousness; (4) wearing a denture. 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 the surrounding areas in each direction. Eligible participants in each sub-district were then randomly selected and invited to meet the investigators at a sub-district health center. All participants provided written informed consent before taking part in the study.

Assessment of mixing ability

The mixing ability test measures how well a participant mixes a two-colored chewing gum, which consists of the ''Hubba-Bubba Tape Gum'' (The Wrigley Company Ltd., England) used for testing. The test piece measured 30 mm x 18 mm x 3 mm and was made from the strips of the 'Sour Berry' (azure color) and 'Fancy Fruit' (pink color) gum. Strips of two-colored chewing gum were cut from both colors and manually stuck together.[9]

All the participant were examined and instructed by one operator. They were asked to sit upright with their heads in a natural position. Each participant was told to

chew one sample of gum on their preferred chewing side for 20 cycles and to terminate the act of chewing with their mouths closed. After that, the instructor had the participant open their mouths and remove the gum bolus with an explorer. Both the participant and the instructor counted the number of chewing strokes. The gums were then spat into transparent plastic bags and flattened to a wafer of 1 mm thickness and labeled with numbers.

The scanning took place within 24 hours, as the color of the chewing gum might degrade (saliva enzymes). Both sides of the samples were scanned using a flatbed scanner (resolution 300 dpi, Epson Perfection V750 Pro, Seiko Epson Corp., Japan) and subsequently copied into one image. The compound images were then assessed with a purpose-built program, which is freely available (ViewGum# software, dHAL Software, Greece, www.dhal.com). The variance of the hue (VOH) with no measurement unit is considered as the measure of mixing; inadequate mixing presents larger variance on the hue axis than complete mixing. The method used was originally described by Halezonetis *et al.*[10]

Oral Examinations

Examinations of periodontal status dental caries and posterior occlusal contact were performed in the sub-district health center using mouth mirror and a periodontal probe. The participants were examined on a 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.[11] The examiner was well-calibrated with a periodontist. The 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. Posterior occlusal contact was recorded according to the Eichner Index[12], based upon existing contact points of natural teeth between the maxilla and mandible in the bilateral premolar and molar regions. According to the Eichner Index, the molar and premolar occlusal contacts of the residual teeth defined the classification. Group A had contact in four support zones; group B had one to three zones of contact or contact in the anterior region only; and group C had no occlusal contact at all, although a few teeth could remain.

Stimulated salivary flow rate

Before oral examination and mixing ability test stimulated whole saliva was collected by the mastication method. The subjects were asked to swallow all the saliva in their mouths, chew a measured amount of paraffin wax (Orion Diagnostica, Finland) for 5 minutes at their own pace and then spit into a graduated tube. After collection, flow rates of the whole saliva were expressed as mL/min. Subjects were classified into two groups according to their salivary flow rates. Subjects whose stimulated salivary flow rate was less than 0.5 mL/min were placed in the hyposalivation group, and the remaining subjects were designated as the normal salivary flow group.[13]

Statistical Analysis

Data are expressed as means and standard deviations (SDs) for continuous variables and as frequencies and percentages for categorical variables. Data was analyzed using IBM SPSS software version 19.0 (SPSS, Chicago, IL, USA). The periodontal status was assessed in the extent of periodontitis based on the percentage of sites with CAL ≥ 5mm which was obtained from (the total site of periodontal pockets with CAL ≥5mm / total number of probed pockets) x 100. Pearson correlations were used to test the association of percent of CAL ≥ 5 and salivary flow rate with mixing ability. The independent *T*-test was used to test for differences in mixing ability among socioeconomic status and health behavior, and One-Way ANOVA was performed to test for differences in mixing ability among subgroups A, B, C according to the Eichner Index. A multiple linear regression analysis was carried out to test the relationship of each explanatory variable with the outcome variable (mixing ability: The

variance of the hue (VOH)) after controlling for the other factors. With the explanatory variable, the salivary flow rate and periodontal status based on the percentage of sites with CAL≥5 mm were used as continuous variables. Gender had only two categories and was scored as female = 0, male = 1. Posterior occlusal contact had three categories, from which dummy variables were created.

Results

Characteristics of the older participants

There were 120 older participants in this study, with an average age (SD) of 70.2 (6.7) years old. The oldest was 89 years old. The majority of participants were female (59.6 %), married (55.1 %), having a primary school education (60.5 %), and living with family (93.8 %). More than half (65.0 %) were not working, 17.5 % worked in agriculture, and the rest either had their own business or were employed (Table 1). Most participants were non-smokers and non-alcohol drinkers. Only 29.2 % were free of systemic diseases. Regarding oral health behaviors, 94.2 % of the participants brushed their teeth daily, and 75.8 % used fluoride toothpaste. More than 80 % had previous dental treatment, but only 43.3 % had an annual dental visit (Table 2).

Table 1 *Characteristics of Study Participants (n = 120)*

Characteristic	N (%)
Gender	
– Male	49 (40.8)
– Female	71 (59.2)
Age in years	
– 60-69	59 (49.2)
– 70-79	46 (38.3)
– ≥80	15 (12.5)
Marital status	
– Not married	56 (46.7)
– Married	64 (53.3)
Education	
– No formal education	8 (6.7)
– Primary school	70 (58.3)
– Secondary school or higher	42 (35.0)
Occupation	
– Not working	78 (65.0)
– Employed	8 (6.7)
– Agriculture	21 (17.5)
– Business	13 (10.8)
Living arrangement	
– Alone	10 (8.3)
– With children	39 (32.5)
– With spouse	57 (47.5)
– With relatives	14 (11.7)

Table 2 *Health Behavior Information (n = 120)*

Characteristic	N (%)
Current smoking	
– Yes	21 (17.5)
– No	99 (82.5)
Alcohol drinking	
– Yes	46 (38.3)
– No	74 (61.7)
Presence of systemic disease	
– Absent	35 (29.2)
– Present	85 (70.8)
History of dental treatment	
– No previous treatment	24 (20.0)
– Had previous treatments	96 (80.0)
Annual dental visits	
– No	68 (56.7)
– Yes	52 (43.3)
Frequency of tooth brushing	
– Less than once daily	7 (5.8)
– At least once daily	113 (94.2)
Type of toothpaste	
– Fluoride toothpaste	83 (75.8)
– Non-fluoride toothpaste	29 (24.2)

The average mixing ability was 0.14 (SD = 0.12, min = 0.016, max = 0.659). The prevalence of caries experience (DMFT>0) among older people was 81.3 %, with the average DMFT of 15.8 (SD = 7.1). The average number of remaining teeth was 21.6 (SD = 2.9). Regarding periodontal status, the participants had an average CAL of 4.3 (SD = 1.7) mm. Most participants (88 %) had at least one site with CAL≥5 mm. The average percentage of the site with CAL ≥5 mm was 35.8 (SD = 30.1). The average stimulated salivary flow rate was 0.78 (0.32) ml/min. When the participants were classified based on the posterior occluding teeth according to Eichner's index, the majority was subtype B (55.8 %) followed by subtype A (32.5 %) and subtype C (11.7 %) respectively.

Tables 3 and 4 show the bivariate analysis of factors related to mixing ability with Variance of Hue as the outcome. Mixing ability was significantly associated with gender, marital status, posterior occluding teeth according to Eichner's index, percentage of CAL ≥ 5, and salivary flow rate.

Table 3 *Factors Associated with Mixing ability among older people in Khon Kaen Province*

Characteristic	Mixing ability (Variance of Hue) Mean (SD)	*P*-value
Gender		
– Male	0.11 (0.09)	**0.004**
– Female	0.17 (0.12)	
Marital status		
– Not married	0.18 (0.14)	**0.004**
– Married	0.11 (0.09)	
Dental caries		
– No	0.16 (0.12)	0.43
– Yes	0.14 (0.12)	
Eichner ‘s index		
– Eicher A	0.082 (0.066)	**< 0.001***
– Eicher B	0.161 (0.123)	
– Eicher C	0.266 (0.138)	
Annual dental visits		
– No	0.16 (0.12)	0.29
– Yes	0.13 (0.12)	
Presence of systemic disease		
– Absent	0.14 (0.12)	0.99
– Present	0.14 (0.12)	
Frequency of tooth brushing		
– Less than once daily	0.15 (0.14)	0.49
– At least once daily	0.13 (0.10)	

Boldface indicates statistical significance.

**Statistically significant differences were found at each comparison of Eichner subgroup, One way ANOVA test*

Table 4 *Factors Associated with Mixing ability among Elderly in Khon Kaen Province in Pearson’s correlation*

Factors	Mixing ability	Percent of CAL ≥ 5	Salivary flow rate
Mixing ability			
R			
***P*-value**			
Percent of CAL ≥ 5			
R	0.305		
***P*-value**	**0.001**		
Salivary flow rate			
R	- 0.303	- 0.090	
***P*-value**	**0.002**	0.368	

R signifies a Pearson correlation coefficient, Boldface indicates statistical significance

Table 5 presents the multivariate linear regression analyses of the associations of mixing ability with other factors. The results showed that mixing ability was significantly associated with the posterior occluding teeth according to Eichner's index (beta: 1.041, 95% CI: 1.022 – 1.06), percentage of CAL ≥ 5 (beta: 1.003, 95% CI: 1.002 – 1.004) and gender (beta: 1.173, 95% CI: 1.104 – 1.242) but, salivary flow rate lost the significant association with mixing after controlling for other factors.

Table 5 *Associations between mixing ability and factors among older people in Khon Kaen province in multivariate multiple linear regression analysis for mixing ability*

Factors	Beta (95% Confidence Interval)	*P*-value
Eichner 's index	1.041 (1.022 – 1.06)	**0.035**
Percent of CAL ≥ 5 mm.	1.003 (1.002 – 1.004)	**0.029**
Salivary flow rate	0.896 (0.836 – 0.956)	0.073
Female	1.173 (1.104 – 1.242)	**0.022**

Boldface indicates statistical significance.

Dependent variable: mixing ability.

Beta signifies a standardized partial regression coefficient, which indicates the relative importance of each variable.

Percent of CAL ≥ 5 mm, Salivary flow rate were used as continuous variables.

Gender: female = 1, male = 0 (male was the reference group)

Eichner's index: subtype A = 0, subtype B = 1, subtype C = 2 (subtype A was the reference group)

Discussion

Studies reported that factors that affect masticatory performance are associated with a decline of oral health condition[14-17], which seem to accelerate dysfunction with aging. To our knowledge, this was the first epidemiologic study investigating the association between mixing ability and oral status among Thai older people. Our results support the hypothesis that mixing ability dysfunction associated with a decreased condition of oral status. The present study found that persons subjected to low mixing ability have severe attachment loss and less occlusal supporting zone than those with high mixing ability in which potential confounders such as gender and salivary flow rate could be controlled.

The major strength of this study is that the main outcome variable was determined by measuring the mixing ability, which is now widely used, and to evaluate the ability to mix and knead a food bolus. Two-color chewing gum[8,9,18-20] has been used for testing for the quantification of masticatory performance among older people. Validity and reliability studies have shown that mixing ability tests are a reliable alternative to comminution tests.[21,22] CAL used for evaluating periodontitis, which is a disease that can newly develop, regress, or progress over time. As an attachment loss requires long periods to develop[23], the CAL measurement can support the validity of periodontitis case ascertainment. A 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.[11] The Eichner's index was used to classify the occlusal supporting zones that consist of a pair of permanent teeth in the molar and premolar areas.[12] These areas are very important to support for chewing food. Having no occluding support area will directly affect the efficiency of chewing ability. Posterior occlusal contacts of the remaining teeth have been

confirmed as key predictors of the reduction of masticatory performance in an earlier study.[24] The previous study has demonstrated that replacing missing teeth with a removable prosthesis cannot approach the efficiency of a complete natural dentition.[25] These reveal that the preservation of posterior functional teeth may be of primary importance for masticatory performance. Several studies provided evidence that masticatory performance was associated with tooth loss.[26] The present study also showed that the number of remaining teeth according to Eichner's index is associated with mixing ability and more strongly in Eichner group A than in groups B and C. These results suggest that the preservation of posterior functional teeth may be of primary importance for masticatory performance.

The results of our study showed the deficient mixing ability among those who have severe periodontitis, which is similar to the study in patients with various periodontal conditions. The periodontal status ranging from healthy to generalized disease categorized by the alveolar bone height–to–tooth length evaluated the effect of periodontitis on masticatory performance revealed that the masticatory performance had a significant correlation with the alveolar bone height. Therefore, the loss of periodontal supporting structures has negative effects on masticatory performance.[27] Natural teeth are equipped with extremely sensitive tactile sensors – periodontal mechanoreceptors situated in the periodontal ligament provide detailed information about intensive and spatial aspects of tooth loads, which support the neural control of masticatory forces.[28-30] Reduced periodontal tissue support accompanies impaired regulation of masticatory forces. Faulty mechanoreceptive innervation of the periodontal ligament and a change in biting strategy due to the weakened support of the teeth may account for the more defensive food-splitting behavior.[31]

Mouth dryness is a common complaint amongst older people and is often associated with diseases and therapeutic medication[32], which suggests hyposalivation is not a physiological, but rather a pathological, age-related characteristic. The association showed that the stimulated salivary flow rate was significant with masticatory performance in the bivariate analysis by lost significance in linear regression analysis after controlling for other factors. Additional analysis was done, and there was a low prevalence of low stimulated saliva flow with 30 % of the study population, which could affect the power of test when adjusted in the final model. The results implied that xerostomia and other dysfunctions related to salivary supply might negatively influence the masticatory process by making it impossible for participants to gather food into a bolus before swallowing.[33] An experimental study showed that the masticatory ability of 15 nondysphagic volunteers aged 22–31 years with natural dentitions was not influenced by experimental oral dryness. The study of 328 independently living people over the age of 60 years also suggested that salivary flow rate is not linearly associated with masticatory performance.[6]

As in other investigations showed inconsistent results, some studies found that gender did not affect masticatory performance[34] but, the study in Japan revealed that the occlusal force in females was significantly lower than in males.[26] Results of this study indicate that the mixing ability in females was significantly lower than in males. Additional analysis was conducted to assure the confounding effect of age, and there is no statistically significant difference between male and female with an average age of 70.8 years and 69.7 years old, respectively. This finding is consistent with mastication; females might compensate for their low muscle strength by increased coordination of other motor and sensory functions.

The findings reported in our study does not establish causality. The 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. The measurement results of mixing ability test with two-colored chewing gum will tell the effect of the ability to mix the colors of the gum in term of "variance of hue." The result with inadequate mixing ability presents with larger variance on the hue than

complete mixing. However, there is still no cut off point that indicates the level of sufficient mixing ability. As there are many factors related to mixing ability and answering such questions requires further study or additional measurement. Our study was limited to the younger side of the old – middle old (60 to 79) group; the mean age of this study population is 70.2 ± 6.7 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 differently from the oldest side of the old group (85+).[35] These may be prone to non-response bias; therefore, the results cannot be automatically applied to the oldest side of the old groups. Suggestions for future research are to increase the number of participants and collect more information which would allow for analysis of possible confounding factors. In the future, longitudinal studies are needed to confirm the causal relationship between oral conditions and mixing ability.

Conclusions

This study provides some evidence of an association between mixing ability and oral status in terms of periodontitis and posterior occlusal contact in older adults when potential confounders, including gender and salivary flow rate, were controlled. Although our results cannot prove a causal relationship, these findings might have valuable implications for the prevention of oral disease and declining of mixing ability in older people.

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Original Article

Sweet Difference Threshold of Strawberry-Flavored Carbonated Drink in 8-Year-Old Children in Khon Kaen

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Abstract

Carbonated drinks are the most popular sugar-contained beverages which might be one of the causes of excessive sugar consumption in Thai children. Stepwise sugar reduction technique, in which the sugar concentration no greater than a person's difference threshold is gradually reduced, is among one of the strategies used to assist people to reduce their sugar consumption. The objective of the present study was to determine the sweet difference threshold of a carbonated test drink in 8-year-old children. Paired-comparisons, forced-choice tests and survival analysis were used in determining the sweet difference threshold of 64 school children. Demographic data, sweet snack and beverage consumption were collected by a questionnaire. The results showed that the overall sweet difference threshold was 15 %. No significant difference in sweet difference threshold was found between gender, areas of school (municipal vs non-municipal), parents' education, family income, frequency of sweet snack and beverage consumption, frequency of strawberry-flavored-carbonated-drink consumption and frequency of adding sugar in their food. The threshold was greater than that obtained from a non-carbonated drink reported previously, indicating the possible effect of carbonation on sweet perception. The threshold value could be used to set the percentage sugar reduction steps in the stepwise sugar reduction program.

Keywords: Carbonated drink, Difference threshold, Sugar consumption, Sugar reduction

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Introduction

Excessive sugar consumption is a risk factor of non-communicable diseases (NCDs) and dental caries. World Health Organization (WHO) has recommended daily intake of free sugar below 10 % or 5 % of total energy intake (i.e. 25 grams) per day for more benefits.[1] In Thailand, the Ministry of Public Health recommends less than 6 teaspoons (or 24 grams of sugar) per day. Despite these recommendations, Thai people have been

consuming sugar approximately 4 times higher than that suggested by WHO. Thai children aged below 5 years have daily sugar intake of 30.4 grams which is equivalent to 7.6 % of total energy intake.[2]

According to Food Consumption Data of Thailand, (2016) the most popular sugar-contained beverage was carbonated drink. The data showed 70.9 % of children aged between 6-12.9 years consumed 300 ml of carbonated drink per day on average.[3] A regular size of carbonated drink (330 ml) contains approximately 30 grams of sugar, the amount of which already exceeds the daily recommendation. Stepwise sugar reduction technique is among one of the strategies used to assist people to reduce their sugar consumption.[4,5] The technique employs the window of sugar concentration where a subject can 'just' distinguish the difference in sweetness, so called 'sweet difference threshold'. In order to gain the most benefit from this technique, the actual difference threshold should be used in the determination of concentration intervals to be gradually reduced. Using chocolate milk[5] and fruit juice[6,7], it has been demonstrated that the average sweet difference threshold in children aged 6-12 years is 11.36 % which is significantly larger than that of adults[6], indicating that children are less sensitive to changes in sugar concentration. On the other hand, the sweet difference threshold of carbonated drinks has never been studied but it is speculated that the carbonation could affect the perceived sweetness. The objective of the present study was to determine the sweet difference threshold of a carbonated test drink in 8-year-old children. The strawberry flavor was used since the color and taste can be easily controlled.

Materials and Methods

This cross-sectional study was approved by the Ethical Committee at Khon Kaen University (HE 612331) and conducted between February–March 2019. Sixty-six children aged 8 years to 8 years 11 months on the date of data collection (February 1st, 2019) from the elementary schools in Muang district, Khon Kaen province participated in the study. The sample size was based on the minimum number of subjects conventionally employed during taste threshold studies, plus 10 % of subjects who might be excluded due to their taste insensitivity during the 50 % sugar difference test.[8] The schools were selected using a stratified randomization method. Included subjects were healthy children who consumed any carbonated drink at least once a week. Excluded subjects were those who strongly disliked strawberry flavor, had diseases affecting taste and smell perception, were presently on sugar dietary control, were on continued medication during past 3 months, and could not distinguish the sweet difference between 2 concentrations of strawberry-flavored carbonated test drink with 50 % sugar difference. Assents were given by children and consents were given by their parents. Subjects were asked to fill a questionnaire, consisting of age, gender, family income, parents' education, frequency of sweet snack and beverage consumption, frequency of strawberry-flavored soft drink consumption and frequency of adding sugar in their food.

Preparation of test solutions

Seven sugar concentrations of strawberry-flavored carbonated test solutions were prepared from a fixed proportion of 3 ingredients: 80 %v/v of soda water (Rock Mountain®, Thai Beverage Marketing, Bangkok, Thailand), 9 %v/v of concentrated artificial strawberry flavored syrup (Hale's Blue Boy, Hale's Trading, Thailand) (containing 78 %w/v of sucrose), and 11 %v/v of varying concentrations of sucrose solution (KBS First, Kornburi Sugar, Nakorn Ratchasima, Thailand). Details of sucrose concentration of prepared strawberry-flavored carbonated test solutions were shown in Table 1.

Table 1 *Detailed concentrations of test solutions and the amount of added sugar.*

No.	Sugar reduction percentage from the reference solution (%)	Sugar concentration in test solutions (%w/v)
0 (Reference)	0	14.00
1	9	12.74
2	12	12.32
3	15	11.90
4	18	11.48
5	21	11.06
6	24	10.64

The reference sugar concentration used in this study was 14 % similar to that contained in a commercial strawberry-flavored carbonated drink available in Thailand. The percent reduction of sucrose concentration relative to the reference solution was obtained by a pilot study and set as 9 %, 12 %,15 %, 18 %, 21 % and 24 % respectively.

The syrup and sucrose solution were pre-mixed, stored in a refrigerator at 4±1°C and used within 24 hours. Before the test, 4 ml of the mixture was transferred to each plastic cup and 16 ml of soda water was added to give the final volume of 20 ml. A bottle of soda water was kept in 6°C iced water and used within 20 minutes after being opened in order to minimize the loss of carbon dioxide. All test solutions were served at 10°C in monadic sequence, in random order, to avoid carry-over effects. All temperatures were controlled using a thermometer.

Experimental procedure

Participants were tested one at a time, in a quiet room either in the morning or the afternoon, at least one hour before or after lunch. Each participant was seated face-to-face with the examiner and was instructed about the procedure before starting the experiment. To determine the sweet difference threshold, a paired-comparisons, forced-choice method was conducted. The procedure started with familiarization trials. First, the participants were requested to taste a pair of test solutions having the same sucrose concentration (14 %) and asked to choose the sweeter one, even they were not be able to detect the difference. Second, a pair of 14 % and 7 % sucrose solutions (50 % difference in concentration) was tested and the participants were again asked to choose the sweeter one. Between each trial, participants rinsed their mouth with 20-ml of drinking water. Participants who were not cooperative or unable to detect the difference between the second pair of test solutions (50 % difference of sugar concentration) were excluded from the study.

After the familiarization test, participants assessed 6 pairs of test solutions. Each pair contained a cup of 20-ml reference concentration and a cup of 20-ml reduced-sugar solution. served in a random order. The participants were asked “which one is sweeter?”. The results were then recorded by the examiner. Between each trial, the participants rinsed their mouth with a cup of 20-ml drinking water, followed by a 30-s break. The next pair of test solutions was then evaluated in the same manner until all 6 pairs of solutions were finished. The correct answer (reference solution was sweeter) was recorded as ‘YES’ whereas the incorrect answer recorded as ‘NO’. The lowest sucrose concentration in which a participant consistently and correctly detected the difference were recorded as the sweet difference threshold of that individual.

Statistical analysis

The average age of participants was shown by mean and standard deviations. The other demographic data was described in frequency and the distribution between group were then compared by using Chi-square test (significance level = 0.05).

A survival analysis (Kaplan Meier estimate) was conducted to determine the overall sweet difference threshold of all participants and compared between groups of participants.[6,7]

Results

Among 66 participants, 33 (17 boys, 16 girls) were students from the schools in the municipal area. The mean age of all participants was 8.43±0.25 years. There were no significant differences in the distribution of age, gender, family income, parents' education, frequency of sweet snack and beverage consumption, frequencry of strawberry-flavored soft drink consumption and frequency of adding sugar in their food between participants from municipal and non-municipal schools (Table 2).

Two of the 66 participants failed the 50 % sugar concentration difference test, resulting in 64 participants in the subsequent test for difference threshold. The overall sweet difference threshold was 15 %. The medians of sweet difference threshold were 15 % in boys and 18 % in girls. In addition, 10.6 %, 18.2 %, 21.2 %, 22.7 %, 21.2 % and 3.0 % of participants were able to correctly distinguish between the reference solution and 9 %, 12 %, 15 %, 18 %, 21 % and 24 % reduced-sugar solutions respectively (Table 3). No significant difference in the threshold was found between school area, gender, household income, parents' education, frequency of sweet snack and beverage consumption, frequency of strawberry-flavored soft drink consumption and frequency of adding sugar in their food (Table 4). The results of Kaplan Meier survival analyses were shown in Figure 1.

Discussion

The procedure used to determine the sweet difference threshold in this study was adapted from previous studies which used paired-comparisons, forced-choice technique to determine the taste perception in both children and adults.[5-7,9] The technique has been claimed as a proper method for children.[9,10] The familiarization test was performed prior to the actual experiment to allow the participants to be familiar with the method. Most participants were co-operative and able to follow the given instructions. Only 2 of 66 participants failed to detect the difference between the reference and the 50 % reduced-sucrose solution.

In the present study, the sweet difference threshold of the carbonated drink was found to be 15 %, meaning that 50 % of participants were able to detect the difference of sweetness when sugar concentration was reduced up to 15 % from the reference concentration. Our value was larger than 10 % reported by Lima *et al.*,[6] who tested the difference threshold of grape juice in children. Although the reference concentration used in Lima's study was lower (10 % compared to 14 % in the present study), our difference threshold would not be affected according to Weber's law which states that the ratio between the detectable difference and the initial stimulus intensity is constant.[11] The difference was probably due to the effect of carbonation. The fizziness of soda water might interfere with the sweet taste transduction mechanism and decrease taste sensitivity.[12] Another factor that might affect the threshold value was the cold temperature of our test solutions. However, both reference concentration and the serving temperature used imitated those in the commercially available carbonated drink.

The sweet detection threshold in Thai children has been studied and reported to be 25.9 mM or around 0.89 % (w/v).[13] The detection threshold was not significantly different between boys and girls, and was not associated with sweet preference. It was speculated that sweet detection threshold was dependent of a child's innate capacity, in contrast to sweet preference which was likely to be a learned experience.[14] The sweet difference threshold, on the other hand, has been less studied and was reported to be large in children and decreased in adults.[6] We could not demonstrate any association sweet difference threshold and sweet consumption behavior. This could be because the ability to distinguish taste intensity was affected by multifactorial factors[9,15-17] and probably was dependent of taste practice rather than its exposure. The inability to see any association could also be due to the small sample size of the present study.

In conclusion, under the limitation of the study, we have determined for the first time, the sweet difference threshold of carbonated drink in children. The threshold was greater than that tested with non-carbonated fruit juice. The value could be used to determine the sugar reduction steps during a stepwise (gradual) sugar reduction program.

Table 2 *Demographic data of the participants.*

	Municipal schools	Non-municipal schools	Total	*p*-value
Gender				
Boys	17 (51.5 %)	16 (48.5 %)	33 (50 %)	1.000
Girls	16 (48.5 %)	17 (51.5 %)	33 (50 %)	
Parents' education				
<high school	15 (48.4 %)	23 (69.7 %)	38 (59.4 %)	0.126
≥high school	16 (51.6 %)	10 (30.3 %)	26 (40.6 %)	
Household income				
≤20,000 Baht/month	29 (87.9 %)	30 (90.9 %)	59 (89.4 %)	0.500
>20000 Baht/month	4 (12.1 %)	12 (9.1 %)	7 (10.6 %)	
Frequency of sweet snack and beverage consumption				
≤2 times/day	15 (45.5 %)	11 (33.3 %)	26 (39.4 %)	0.225
>2 times/day	18 (54.5 %)	22 (66.7 %)	40 (60.6 %)	
Frequency of strawberry-flavored soft drink consumption				
≤1time/week	15 (45.5 %)	12 (36.4 %)	27 (40.9 %)	0.617
>1time/week	18 (54.5 %)	21 (63.6 %)	39 (59.1 %)	
Frequency of adding sugar in food				
≤1time/week	21 (63.6 %)	23 (69.7 %)	44 (66.7 %)	0.794
>1time/week	12 (36.4 %)	10 (30.3 %)	22 (33.3 %)	

Table 3 *Number and percentage of participants who were able to distinguish the difference in sugar reduction.*

Percentage sugar reduction	Number of participants	% of participants	Valid %	Cumulative %
9 %	7	10.6	10.9	10.9
12 %	12	18.2	18.8	29.7
15 %	14	21.2	21.9	51.6
18 %	15	22.7	23.4	75.0
21 %	14	21.2	21.9	96.9
24 %	2	3.0	3.1	100.0
Excluded	2	3.0		
Total	66	100.0		

Table 4 *Sweet difference threshold classified by demography and sweet consumption behaviors.*

	N	Difference threshold	95 % confidence interval	*p*-value
Overall	64	15 %	13.379-16.621	-
School area				
Municipal	32	15 %	12.783-17.217	0.368
Non-municipal	32	15 %	12.629-17.371	
Gender				
Boys	32	15 %	12.700-17.300	0.230
Girls	32	18 %	16.504-19.496	
Parents' education				
<high school	37	18 %	16.279-19.721	0.375
≥high school	25	15 %	11.352-18.648	
Household income				
≤20,000 Baht/month	58	15 %	13.134-16.866	0.361
>20,000 Baht/month	6	15 %	12.737-17.263	
Frequency of sweet snack and beverage consumption				
≤2 times/day	24	15 %	12.950-17.050	0.647
>2 times/day	40	15 %	12.521-17.479	
Frequency of strawberry-flavored soft drink consumption				
≤1 time/week	26	18%	16.338-19.662	0.620
>1 time/week	38	15%	13.373-16.627	
Frequency of adding sugar in food				
≤1 time/week	42	15 %	13.276-16.724	0.250
>1 time/week	22	18 %	14.209-21.791	

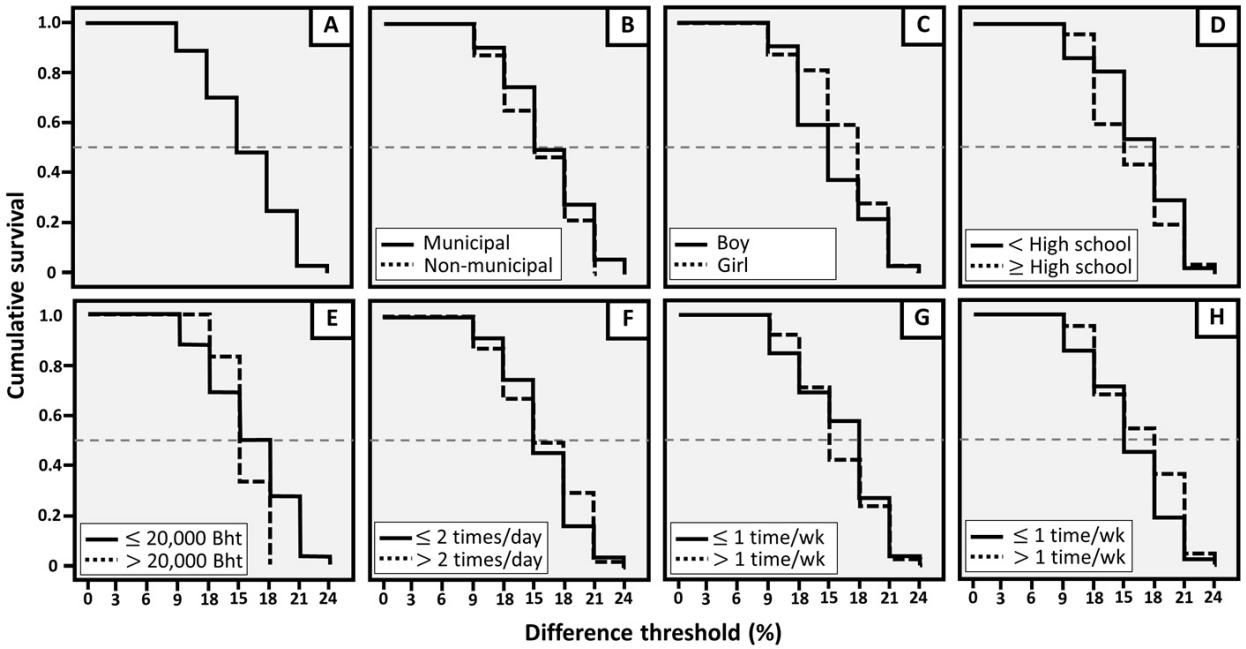

Figure 1 *Meier survival analyses showing the difference threshold of all samples (A) by school area (B), gender (C), parents' education (D), household income (E), children's frequency of sweet consumption (F), frequency of strawberry-flavored soft drink consumption (G), and frequency of adding sugar in food (H).*

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