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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 16th National Scientific Conference of Dentistry (DFCT2017) .

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

On behalf of the President of Dental Association of Thailand I would like to express my sincere appreciation for all the efforts done by DFCT through the passing 35 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 16th National Scientific Conference of Dentistry is another big step of DFCT to promote the awareness and importance of regular participation in the Scientific conference. Thus will enable all the faculties to refresh and produce newly interesting topics in Dental Sciences and enhances the high capabilities of all faculty members, especially the young generation.

To conduct such an important event, the staffs and team work of the host are the crucial issue that will create success of the Conference. I would like to congratulate the Faculty of Dentistry, Prince of Songkla 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 Prince of Songkla Universty.

Dr.Adirek S.Wongsa

President

Dental Association of Thailand

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วิทยาสารทันตแพทยศาสตร์

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Contents

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Original Article

Cytotoxicity of Silver Nano-prisms Containing Acrylic Denture Soft Liners

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Abstract

It is well established that silver compound and its derivatives have been used as an effective antimicrobial agent, although there are concerns that it may have an effect of cytotoxicity on fibroblast cells. This study evaluates cytotoxicity effects of acrylic denture soft liner with silver nano-prisms (AgNPs). Various concentrations (10, 20, 30, 40, 50 and 60 ppm) of AgNPs were incorporated into the acrylic denture soft liner, and divided into seven groups (one control group and six experimental G1-G6 groups). The cytotoxicity of all groups was tested using a duplicate MTT assay. The results were analyzed using a two-way ANOVA with Tukey HSD post-hoc tests ($p<0.05$). Among the concentrations of AgNPs tested at 24 hours, G6 yielded the lowest cell viability (73.25 %) while G1 yielded the highest viability value (98.32 %). The results therefore reveal good cell viability values with an acrylic soft liner and silver nano-prisms. In addition, the experimental acrylic denture soft-lining materials with low concentration of AgNPs is not harmful toward fibroblast cells.

Keywords: Silver nano-prisms, Cytotoxicity, Soft liner

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Introduction

After long-term use of a dental prosthesis, the mucosa under the denture base becomes thinner with severe alveolar ridge resorptions. Patients show an intolerance to the hard denture base when they engage in regular chewing behavior throughout the day. Soft-lining materials are most commonly used for patients who are intolerant to the pressures transmitted by the prosthesis. Soft-lining materials help to enhance the recovery of the denture bearing area when dental trauma occurs to the alveolar ridge and becomes more accurately adapted to the edentulous ridge.[1]

Candida albicans which is considered to be the most important etiological factor in denture stomatitis, can be found on the soft-liner surface.[2,3] Moreover, *in vitro* experiments have shown that none of the materials had an inhibitory effect on the growth of Candida albicans which could penetrate to the inner portions of the soft liner.[4,5]

There are two methods (mechanical and chemical)

for daily denture cleaning. The mechanical method, which uses a toothbrush, will cause damage to the soft liner. For instance, soft-liner materials can become be torn by brushing. Although the chemical method is a better way of cleaning, its' main downside is that chemical cleansers can alter some denture properties such as the loss of softness on the soft-liner materials.[6]

Tissue conditioners act as drug delivery agents to bring antiseptic agents to alleviate denture stomatitis in patients. Antifungal drugs such as nystatin have been commonly used for the treatment of denture stomatitis.[7] However, antifungal drugs have a broad spectrum target in humans which doctors use for treating patients. Although Amphotericin B and nystatin are good antifungal drugs, they are not effective when they are incorporated in the viscogel denture soft liner.[9]

Recently, several new antifungal agents such as metallic oxide powders[9], natural and herbal oils[10], and silver nano-prisms (AgNPs)[11-13,15-16] incorporated in tissue conditioners have been made available for treatment. Silver nano-prisms (AgNPs) have been widely used in clothing, electronics, bio-sensing, the food industry, paints, sunscreens, cosmetics, and medical devices. AgNPs are beneficial because not only do they have the ability to disturb metabolic activity of microorganisms they are also nontoxic to humans. Moreover, AgNPs are used in very low concentrations against microorganisms.[12-14]

Chladek *et al.* (2011) investigated the antifungal activity of denture soft-lining material modified by AgNPs at six concentrations (10, 20, 40, 80, 120 and 200 ppm). They found that the average antifungal efficacy value (AFE) for samples with 40 ppm contained in AgNPs was 31.5 %. Although additional concentrations of AgNPs resulted in a less dynamic outcome, there were still visible increases in AFE values.[15] Consequently, the most appropriate concentration level is 40 ppm.

Chladek *et al.* (2012) found that AgNPs exceeding 80 ppm concentrations could be incorporated to large aggregations, which may decrease their antimicrobial effectiveness due to the reduction of the effective surface area that AgNPs come into contact with microorganisms.[16]

Cytotoxicity tests are an essential screening step for the testing of new materials that are used in humans. They provide a means of investigating toxicity in a simplified system that reduces the effects of toxic variables. The overall purpose of this study was to examine the cytotoxic effects of AgNPs contained on acrylic soft-lining materials. To evaluate the potential of such materials to cause irritation, the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay was applied here. MTT assay offers a high degree of sensitivity while providing a considerable saving in time and labor by eliminating the formazan product prior to absorbance measurements. Thus, it is a quick, easy and safe procedure to perform.[17-18]

Materials and Methods

The materials used in this experiment are

1. Acrylic soft liner materials: Visco-gel Dentsply Co.Ltd, Germany (Table 1 and Fig. 1)
2. Antimicrobial agent: Silver nano-prisms (AgNPs) from Prime NANO technology (Fig. 2)
3. Stainless steel mold: disc-shaped for cytotoxic test (Fig. 3)

Table 1 *Details of materials used in the experiment : Visco-gel®*

Visco-gel	Description
Manufacturer	Dentsply Co.Ltd, Germany
Type	Temporary soft lining materials
Composition	Powder: Polymethyl methacrylate
	Liquid: Phthalyl butyl glycolate, Ethanol
Application method	Powder/liquid : 3 g / 2 ml

Figure 1 *Acrylic soft-liner materials (Visco-gel®, Dentsply Co.Ltd, Germany)*

Figure 2 Silver nano-prisms from Prime NANO tech nology. (Prime Nanotechnology Co. Ltd., Chulalongkorn University, Bangkok, Thailand)

The Visco-gel (acrylic denture soft-lining materials) was prepared by mixing a powder/liquid ratio of 3 g / 2 ml according to the manufacturer's' instructions. Silver nano-prisms were then added into the liquid part of the Visco-gel soft liner, which was divided into seven groups according to the concentration of silver nano-prisms from G1-G6. (Table 2).

Table 2 *The classification of experimental group.*

Sample group	Silver nano-prism concentration (ppm)	Application method Powder (g) : Liquid (ml)	Silver nanoprism Volume (ml)
Control	0	3 : 2.000	0.000
G1	10	3 : 2.002	0.002
G2	20	3 : 2.003	0.003
G3	30	3 : 2.004	0.004
G4	40	3 : 2.005	0.005
G5	50	3 : 2.006	0.006
G6	60	3 : 2.007	0.007

Cytotoxicity test

The cytotoxicity test was administered using an MTT assay. The specimens were divided into seven groups including a control group and six treatment groups. The six treatment groups had a varied concentration of silver nano-prisms (10, 20, 30, 40, 50 and 60 ppm) added to the liquid part of the acrylic soft-lining materials. Each disc-shaped specimen was 10 mm in diameter and 1 mm thick and prepared in a stainless-steel mold (Fig.3). Specimens were grouped in accordance to the incubation period: 0 hour, 24 hour, 48 hours and 72 hours (six specimens for each well plate), and stored at 37°C in an atmoshere of 5 % CO_2.

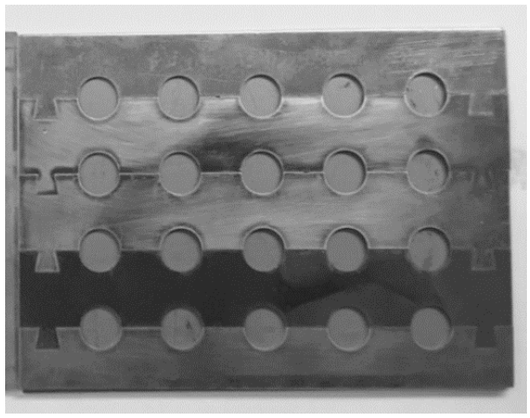

Figure 3 *Stainless steel mold for cytotoxic test specimen fabrication*

Cell culture

L929 mouse fibroblast cell lines in $1x10^5$ cell/cm^3 (ATCC 2869501, obtained from passage 106, ATCC®, USA) were grown as a monolayer culture in a cell culture dish at 37°C in an atm of 5 % CO_2. The culture medium was supplemented with 10 % fetal bovine serum (FBS, HyClone™, South America, USA) and 1 % antibiotic (HyClone™, South America, USA). They were then incubated for 24 hours at 37°C. After 24 hours, the cell culture medium was removed and 200 µL extractions were replaced in a wells plate that were related to the seven test groups. The plates were incubated at 37°C, 5 % CO_2 and 98 % humidity for 24 hours.

Soft liners were mixed according to the manufacturer's' instructions, and then allowed to set at 37°C for 30 minutes. After removal from the molds, the Visco-gel samples were stored in 2 ml of eagle's minimum essential medium (E-MEM, Life Technologies, Inc., Grand Island, NY, USA) without FBS or antibiotics for 1 hour at 37°C. All materials were handled aseptically throughout the procedures described here, before being evaluated by the MTT assay.

MTT assay

The Visco-gel samples incorporated with AgNPs were removed from the wells and replaced with the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) solution diluted in cell culture media. The plates were then incubated for 2 hours at 37°C in 95 % humidity and 5 % CO_2. Following this, formazan crystals were dissolved in dimethyl sulfoxide. The wavelength used for measuring the optical density (OD) of the absorbance on the formazan products was 570 nm as a reference wavelength, by using the Epoch® Spectrophotometer (Vermont, USA). The setting was based on the International Stands Organization (ISO) 10993-5: 2009 specification; all tests were conducted under standardized controlled conditions (95 % humidity and 37°C).

Cell viability and cell cytotoxicity were calculated as a percentage of the control group according to the following modified formula: [17,24,25,26]

$$\text{Cell cytotoxicity (\%)} = 100 - \text{Cell viability (\%)}$$

$$= \left(\frac{\text{OD of the test group}}{\text{OD of the control group}} \right) \times 100$$

Cell viability was scored according to the following classification scale: [17,24,25,26]

- more than 90 % cell viability: non-cytotoxic
- 60–90 % cell viability: slightly cytotoxic
- 30–59 % cell viability: moderately cytotoxic
- less than 30 % cell viability: severely cytotoxic

The dispersion of AgNPs containing soft-lining materials were visualized by a Scanning Electron Microscope

(SEM), (JEOL-6510 LV, JEOL Inc., Tokyo, Japan). The disc-shape specimens were cut and torn at the center before being examined in concentration groups with the SEM using an accelerating voltage of 15 keV.

SPSS version 20.0 (IBM SPSS Inc., Chicago, Illinois, USA) was used to analyze the data in this study. The normality distribution and homogeneity of variance were tested using the Kolmogorov-Smirnov and Levene's test, respectively. Means and standard deviations of the results from the cytotoxicity test were calculated and analyzed statistically using a two-way ANOVA with Tukey's HSD tests used to determine the direction of significance. Alpha was set at 0.05.

Results

The cytotoxicity test of silver nano-prisms (AgNPs) in acrylic denture soft liners were detected by the percentage of optical density in MTT assay after four periods (0hr, 24hrs, 48hrs and 72hrs) and six concentrations of AgNPs (10, 20, 30, 40, 50 and 60 ppm). Mean percentages of optical densities (OD) along with the corresponding standard deviations are shown in Table 3.

Table 3 *Mean percentage of optical density (OD) in MTT assay.*

Concentration of Silver nanoprism (ppm)	Mean percentage of OD ($\bar{X}$(sd))			
	0 hr	after 24 hr	after 48 hr	after 72 hr
Control	99.06 (6.3)	99.16 (6.7)	99.98 (4.3)	99.09 (7.3)
G1 (10 ppm)	98.30 (9.4)	95.77 (5.8)	98.32 (4.1)	98.10 (11.4)
G2 (20 ppm)	94.51 (11.1)	95.41(7.2)	97.35 (6.7)	95.29 (8.1)
G3 (30 ppm)	95.10 (4.5)	90.53 (8.5)	96.65 (4.6)	94.11 (4.0)
G4 (40 ppm)	94.00 (6.1)	81.62 (9.6)	95.58 (8.7)	93.57 (7.0)
G5 (50 ppm)	90.58 (9.4)	74.25 (11.1)	93.74 (4.8)	91.63 (9.4)
G6 (60 ppm)	88.05 (8.8)	73.25 (8.8)	91.53 (5.2)	90.53 (3.3)

A two-way ANOVA was significant ($p<0.05$). The cell viability value was the lowest in the G6 / twenty four hour group (73.25 %), while the highest cell viability value was obtained for the G1 / twenty-four hour group (98.32 %). The lowest of cell viability score which was calculated to the cell cytotoxic score, was less than 30 % of cell cytotoxic scores (that the score was the mean slightly cytotoxic scored ranges).

The SEM images (Fig. 4a-b) show that the control samples do not have any AgNPs particles on the specimens. However, the treatment sample groups do have some AgNPs particles. The highest density of AgNPs is 60 ppm groups as shown in Figure 4 (m) and the lowest density of AgNPs is 10 ppm groups as shown in Figure 4 (c). AgNPs particles were found to be more dispersible in the border area of the specimens compared to other areas.

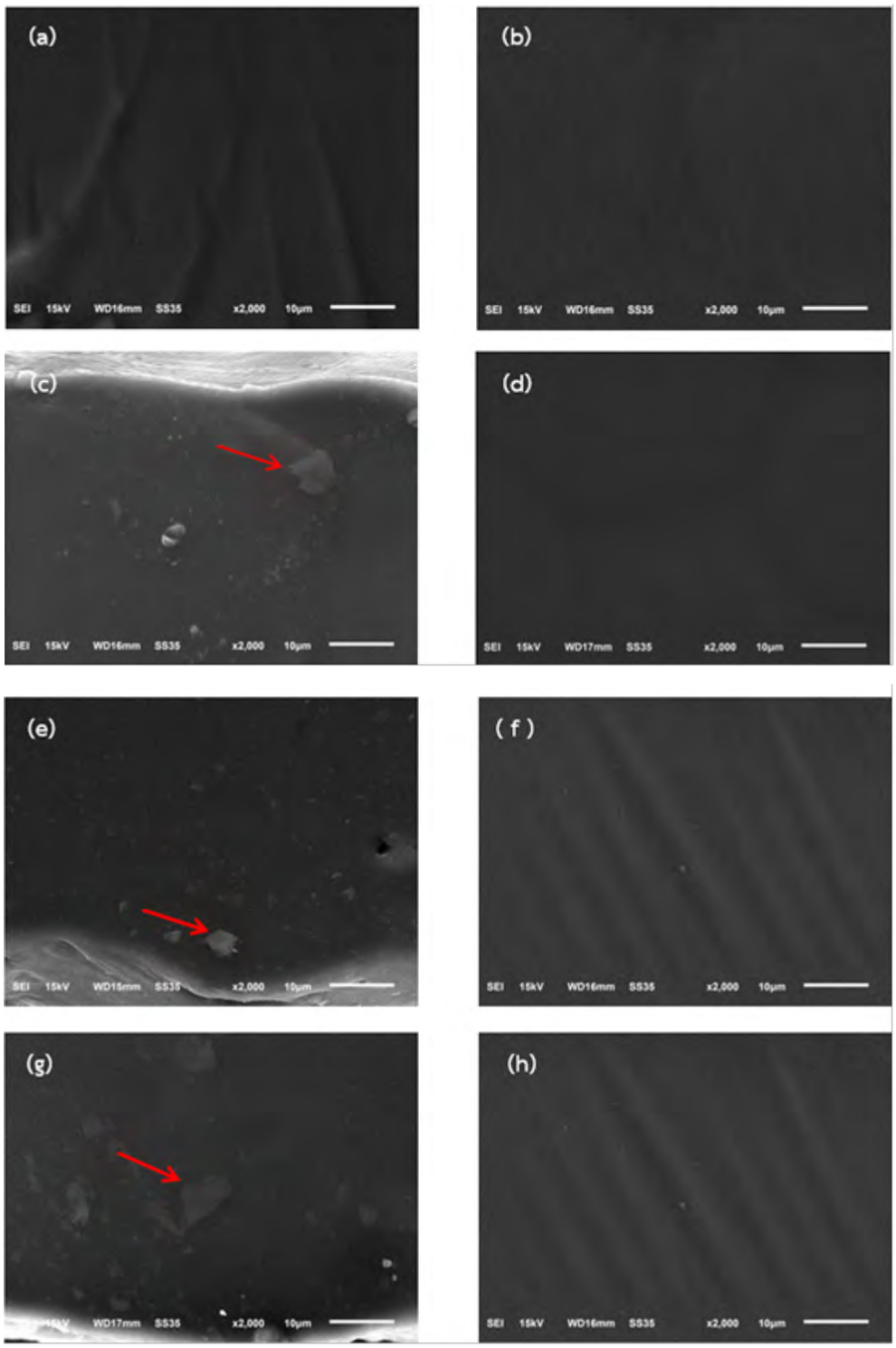

Figure 4 *Scanning electron microscopy image (x2000 magnification) of soft-lining materials specimens. (a) and (b) Control group specimens. (The left side represents the specimen borders; the right side represents the inner part of the specimens). (c) to (n) Treatment group with AgNPs in 10,20,30,40,50 and 60 ppm concentration (the left side represents the specimen border; the right side represents the inner part of the specimen). The red arrows indicate AgNPs dispersed in denture soft-lining materials.*

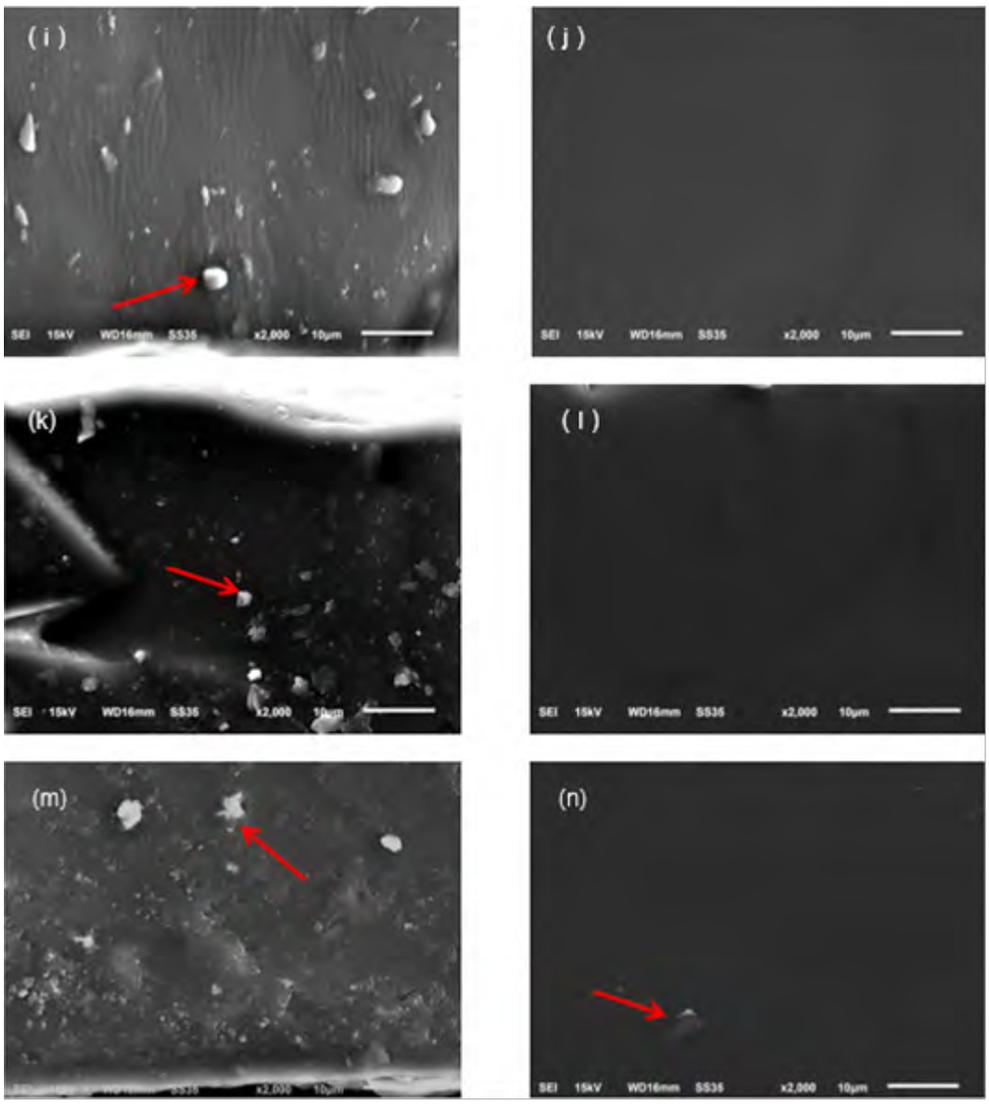

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Figure 4 *Scanning electron microscopy image (x2000 magnification of soft-lining materials specimens. (a) and (b) Control group specimens. (the left side represents the specimen borders; the right side represents the inner part of the specimens). (c) to (n) Treatment group with AgNPs in 10,20,30,40,50 and 60 ppm concentration (left side represents the specimen borders, the right side represents the inner part of the specimens. The red arrows indicates AgNPs dispersed in denture soft lining materials.*

Discussion

A recent study found that AgNPs were added to soft-lining materials to improve the antimicrobial effects of these materials. However, there has not been a study on the toxicity of silver when added to soft-lining materials. This study investigated the cytotoxicity of AgNPs contained in soft-lining materials.

Landayan, *et al* (2014) found that out of four commonly used soft denture lining materials (Coe-Comfort® GC America, USA; Coe-Soft® GC America, USA; Visco-gel® Dentsply, USA; and Sofreliner Tough M®, Tokuyama Dental Corporation, Japan), Visco-Gel® produced the lowest cytotoxicity effects, which is available and used

in Thailand[20], so it was selected for this study in order to compare the cytotoxicity effects of each silver nano-prism concentration in the soft-lining materials that are used in dentures.

Parnklang *et al.* (2013) found that silver nano-prisms made from the rapid thermochemical synthesis of AgNPs employing hydrogen peroxide (H_2O_2), transformed from the shape of silver nano-spheres (AgNSs) to silver nano-prisms (AgNPs). Synthesized AgNPs are stable enough for prolonged storage of around 3-6 months in liquid form, and can be used to manipulate metallic crystals at the nanoscale. The shape of the AgNPs obtained exhibited mixed geometries such as hexagonal, triangular, rounded-tip triangular prisms, and circular disks with average bisector lengths of 30 to 120 nm and a thickness of 10 to 20 nm.[21] A liquid form of AgNPs was selected for this study because it can be more easily dispersed than just in a powder composition of soft lining materials. A liquid form of silver nano-prisms is also stable for prolonged storage of around 3-6 months.

Of all concentrations (G1-G6) in this study, the highest percentage of cell cytotoxicity was found in the 24-hour group (G1-G6). Cell cytotoxicity was found to decrease after 48 and 72 hours of incubation. This pattern of results is compatible with Landayan et al. (2014).[20] Similarly, Jones *et al.* (1988) examined the composition of plasticizer and leachability in dental soft polymers and found that ethyl alcohol completely evaporates within 24 hours from polymer gel materials stored in water at 37ºC, and the plasticizer would increase evaporates from 0.30 mg/g to 8.70 mg/g within 14 days.[22] Therefore, the cell cytotoxicity of soft lining material in the 24-hour group is higher than the others.

Chladek, *et al.* (2011) investigated antifungal activity of denture soft-lining material modified by silver nano-prisms at six concentrations (10, 20, 40, 80, 120 and 200 ppm). They found that the average antifungal efficacy value (AFE) for samples with 40 ppm contained in AgNPs was 31.5. However, additional increases in concentration of AgNPs resulted in less dynamic AFE value outputs, although there were still visible AFE value changes.[15] Consequently, the appropriate strength of AgNPs concentration was the 40 ppm concentration group.

Skladanowski *et al.* (2016) evaluated the cytotoxicity, immune compatibility and antibacterial activity of biogenic silver nano-prisms. They studied the cytotoxicity of AgNPs at concentration levels of 1, 5, 10, 25, 50 and 100 µg/mL (ppm). They found that the half-maximal inhibitory concentration (IC50) value of the cytotoxic test was established at 64.5 µg/mL by MTT assay methods.[23] Similarly, Taleghani *et al.* (2014) examined cytotoxicity of silver nano-prisms on human gingival epithelial cells. The concentration of AgNPs solution used were 0.05, 0.1, 0.2, 0.5, 1, 2, 5, 10, 20 and 50 µg/ml (ppm). They reported that cytotoxicity effects of nanosilver solution occurred at high concentrations (exceeding 50 ppm) but not at lower concentrations (below 20 ppm).[24] Consequently, the appropriate concentration range of AgNPs in the current study was selected between 10 ppm to 50 ppm.

The SEM image showed the diffusion of AgNPs in soft lining materials. The current findings revealed that AgNPs were more dispersible in the border of the materials than in any other area. When soft lining materials containing AgNPs had polymerization, the silver ions were extruded by the polymerize force of materials into the border area. Many patients who use soft-lining materials in the denture base are likely to see improved denture stomatitis because it is necessary to contact the silver nano-prisms ions in the tissue surface of the denture in order to increase their antimicrobial effects.

Conclusion

Our results indicated good cell viability values of acrylic denture soft liner and silver nano-prisms. We have shown that the experimental acrylic soft-lining materials with a low concentration of AgNPs is not

harmful toward fibroblast cells. Silver nano-prisms containing acrylic denture soft liner appears to be a promising and innovative material for use in clinical situations.

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References

1. Wright PS. Soft lining materials: their status and prospects. ***J Dent*** 1976;4:247-56

2. Masella RP, Dolan CT, Laney WR. The prevention of growth of candida on Silastic 390 soft liner for denture. ***J Prosthet Dent*** 1975;33:250-7.

3. Burns DR, Burns DA, DiPietro GJ, Gregory RL. Response of processed resilient denture liner to candida albicans. ***J Prosthet Dent*** 1987;57:507-12.

4. Bulad K, Taylor RL, Verran J, McCord JF. Colonization and penetration of denture soft lining materials by Candida albicans. ***Dent Mater*** 2004;20:167-75.

5. Graham BS, Jones DW, Burke J, Thompson JP. In vivo fungal presence and growth on two resilient denture liner. ***J Prosthet Dent*** 1991;65:528-32.

6. Harrison A, Basker RM, Smith IS. The compatibility of temporary soft materials with immersion denture cleansers. ***Int J Prosthodont*** 1989;2:254-8.

7. Chow C, Matear D, Lawrence H. Efficacy of antifungal agents in tissue conditioners in treating candidiasis. ***Gerodontology*** 1999;16:110–8.

8. Thomas CJ, Nutt GM. The in vitro fungicidal properties of visco-gel, alone and combined with nystatin and amphotericin B. ***J Oral Rehabil*** 1978;5:167–72.

9. Kanathila H, Bhat AM, Krishna PD. The effectiveness of magnesium oxide combined with tissue conditioners in inhibiting the growth of Candida albicans: an *in vitro* study. ***Indian J Dent Res*** 2011; 22:613.

10. Muttagai S, Subramanya JK. Effect of incorporating seed oils on the antifungal property, surface roughness, wettability, weight change, and glucose sorption of a soft liner. ***J Prosthet Dent*** 2017;117:176-84.

11. Issa IM, Abdul-Fattah Nabeel. Evaluating the effect of silver nanoparticles incorporation on antifungal activity and some properties of soft denture lining material. ***J Bagh Coll Dentistry*** 2015;27:17-23.

12. You C, Han C, Wang X, Zheng Y, Li Q, Hu X, *et al.* The progress of silver nanoparticles in the antibacterial mechanism, clinical application and cytotoxicity. ***Mol Biol Rep*** 2012;39:9193-201.

13. Panácek A, Kolár M, Vecerová R, Prucek R, Soukupová J, Krystof V, *et al.* Antifungal activity of silver nanoparticles against Candida spp. ***Biomaterials*** 2009;30(31):6333-40.

14. Jeong SH, Yeo SY, Yi SC. The effect of filler particle size on the antibacterial properties of compounded polymer/silver fibers. ***J Mater Sci*** 2005;40:5407–11.

15. Chladek G, Mertas A, Barszczewska-Rybarek I, Nalewajek T, Zmudzki J, Król W, *et al.* Antifungal activity of denture soft lining material modified by silver nanoparticles-a pilot study. ***Int J Mol Sci*** 2011;12:4735-44.

16. Chladek G, Barszczewska RI, Lukaszczyk J. Developing the procedure of modifying the denture soft liner by silver nanoparticle. ***Acta Bioeng Biomech*** 2012;14:23-9.

17. Stockert JC, Blázquez-Castro A, Cañete M, Horobin RW, Villanueva A. MTT assay for cell viability: Intracellular localization of formazan product is in lipid droplets. ***Acta Histochem*** 2012;114:785-96.

18. Terry LR, Richard AM, Andrew LN, Helene AB, Tracy JW, Lisa M. Cell viability assays guidance manual (internet), 2013 May 1 [Updated 2016 Jul 1]. [cited 2018 April] Available from: https://www.ncbi.nlm.nih.gov/books/NBK144065/

19. International Standards Organization(ISO) 10993-5:1992. Biological evaluation of medical devices- Part5: Tests for in vitro cytotoxicity. International standards organization, Geneva; Switzerland; 2009;3:1-42.

20. Landayan JI, Manaloto AC, Lee JY, Shin SW. Effect of aging on tear strength and cytotoxicity of soft lining materials; *in vitro.* ***J Adv Prosthodont*** 2014;6:115-20.

21. Pamklang T, Lertvachirapaiboon C, Pienpinijtham P, Wongravee K, Thammacharoen C, Ekgasit S. H2O2- triggered shape transformation of silver nanospheres to nanoprisms with controllable longitudinal LSPR wavelengths. ***RSC Adv*** 2013;3:12886–94.

22. Jones DW, Sutow EJ, Hall GC, Tobin WM, Graham BS. Dental soft polymers: plasticizer composition and leachability. ***Dent Mater*** 1988;4:1-7.

23. Składanowski M, Golinska P, Rudnicka K, Dahm H, Rai M. Evaluation of cytotoxicity, immune compatibility and antibacterial activity of biogenic silver nanoparticles. ***Med Microbiol Immunol*** 2016;205:603-13.

24. Taleghani F, Yaraii R, Sadeghi R, Haghgoo R, Rezvani M B.

Cytotoxicity of silver nanoparticles on human gingival epithelial cells: an *in-vitro* study. ***JDS*** 2014;32:30-6.
25. Heravi F, Ramezani M, Poosti M, Hosseini M, Shajiei A, Ahrari F. *In vitro* cytotoxicity assessment of an orthodontic composite containing titanium-dioxide nano-particles. ***J Dent Res Clin Dent Prospects*** 2013;7:192-8.
26. Ahrari F, Afshari JT, Poosti M and Brook A. Cytotoxicity of orthodontic bonding adhesive resins on human oral fibroblasts. ***Eur J Orthod*** 2010;32:688–92.
27. Sjögren G, Sletten GB and Dahl JE. Cytotoxicity of dental alloys, metals, and ceramics assessed by millipore filter, agar overlay, and MTT tests. ***J Prosthet dent*** 2000;84:229-36.

Original Article

Effect of Methyl Formate-methyl Acetate Treatment on the Tensile Bond Strength between Denture Teeth and Denture Base Resin

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Abstract

The aim of this study was to examine the tensile bond strength between two types of acrylic denture teeth and a heat-polymerized acrylic denture base after being treated with acrylic denture base liquid or methyl formate-methyl acetate (MF-MA) solutions. Conventional denture teeth and highly cross-linked denture teeth were polished at the ridge lap surface and then randomly divided into 12 groups. Groups 1 and 7 were control groups. Groups 2 and 8 were treated with acrylic denture base liquid (MMA) and the remaining groups were treated with MF-MA solutions at various concentrations (25:75, 40:60, 55:45, and 70:30 % v/v). Heat-polymerized acrylic resin was bonded to the denture teeth. Small dumbbell-shaped specimens were prepared and tensile bond strength testing was performed. The data was analyzed using two way ANOVA and one way ANOVA where significant differences in the groups were found, individual means were compared with the Tukey test at a 95 % confidence level. The surface treated groups demonstrated higher tensile bond strengths than the untreated groups ($p<0.05$), except for the 70:30 % v/v group. Within each surface treatment group, the type of denture teeth had no effect on bond strength ($p>0.05$). This study suggests the application of acrylic denture base liquid or a MF-MA solution (25:75, 40:60, or 55:45 v/v) before packing the acrylic resin can increase the bond strength between denture teeth and the denture base.

Keywords: Acrylic denture teeth, Acrylic denture base, Tensile bond strength, Surface treatment

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Introduction

Removable dentures are widely used by edentulous individuals. However, common removable denture problems include debonding of the denture teeth and denture fracture.[1] Denture tooth debonding may occur because of contamination of the tooth or denture surface, or differences in the structures of the two components.[2] There are two major methods for improving the bond strength between acrylic denture teeth and the denture base. Mechanical methods include grinding the tooth ridge lap surface[3], preparing a retention groove[4], or sandblasting.[5] Alternatively, chemical methods employ a chemical solution applied at the ridge lap surface. Previous studies have shown that MMA[6-8], MMA-based bonding agent[9], 4-META (4-methacryloxyethyl trimellitate anhydride)[10], dichloromethane (CH_2Cl_2)[11,12], methyl acetate (MA) based experimental bonding agent[13], or tribochemical silica coating and silanization[14] could increase bond strength. However, some studies indicated that MMA did not enhance the bond strength.[15-17]

The use of a chemical agent such as methyl formate (MF), MA or MF-MA solution has been suggested for repairing acrylic denture base resin. These agents reduced adhesive failure compared with the use of MMA.[18] A previous study showed that MF-MA solution enhanced the shear bond strength between reline resins and denture base resin.[19] A comparative study of the effect of these chemical agents on the bond strength between denture teeth and denture base has not yet been reported.

The objectives of the present study were to: 1) evaluate the effect of MMA and MF-MA solutions as surface treatments on the tensile bond strength between acrylic denture teeth and a heat-polymerized acrylic denture base, and 2) compare the bond strength between conventional denture teeth and highly cross-linked denture teeth. The first null hypothesis was that the tensile bond strength of the treated surface denture teeth and denture base resin were not significantly different from that of the untreated surface group. The second null hypothesis was that the tensile bond strength of the conventional acrylic denture teeth groups also were not significantly different from that of the highly cross-linked acrylic denture teeth groups.

Materials and methods

Sixty lower posterior first molar conventional acrylic denture teeth (Major Dent, Major Prodotti Dentari, Moncalieri, Italy) and sixty lower posterior first molar highly cross-linked acrylic denture teeth (Cosmo HXL, Dentsply Dental, Tianjin, China) were polished at the ridge lap surface using a polishing machine (Ecomet 250, Buehler, Illinois, USA) with 400, 800, and 1200-grit silicon carbide paper. Vaseline was applied to the internal surface of a tube (18 mm height, 15 mm diameter), and the tube was placed on a flat metal plate and filled with warmed modeling wax. The denture tooth was placed on surface of the wax, secured with the warmed modeling wax, and the tube/tooth specimen was immersed in cool water. After the wax hardened, the specimen was removed from the tube. Dental plaster was poured into the lower half of a flask. The specimen was placed in the plaster with the tooth and approximately 3 mm of the wax tube was exposed. Separating media was applied to the plaster surface. The upper part of flask was attached and filled with dental plaster. The flask was pressed (2,000 kgf) for 30 minutes. After the plaster set, the flask was placed in boiling water for 5 minutes. The flask was opened and the softened wax was removed by washing with boiling water and anionic detergent. Prior to denture base resin packing, the specimens were distributed into 12 groups (n=10 for each group). Groups 1 and 7 were control groups (no treatment). In the remaining groups, the ridge lap surfaces of the teeth were treated with a chemical agent for 15 seconds: groups 2 and 8 were treated with acrylic denture base liquid, the remaining groups were treated with a MF-MA solution at various

concentrations (25:75, 40:60, 55:45, 70:30 % v/v). The denture base resin (Meliodent, Heraeus Kulzer, Sanden, Germany) was bonded according to the manufacturers' recommendations within 5 minutes after surface treatment. The flasks were then pressed (2,000 kgf) for 1 hour. The specimens were polymerized at 74°C for 9 hours. Subsequently, the flasks were kept in a curing unit until the water reached room temperature.

Table 1 *Materials used in this study*

	Product name	Materials	Manufacturer
denture teeth	Major dent	Conventional polymethyl methacrylate (lot no.9072)	Major Prodotti Dentari, Italy
	Cosmo HXL	Highly crosslinked–IPN polymethyl methacrylate (lot no.20120525D)	Dentsply Dental ,Tianjin, China
denture base chemical solutions	Meliodent	Heat-polymerized acrylic resin (lot no.10NOV087)	Heraeus Kulzer, Sanden, Germany
	Meliodent (liquid)	Liquid of Heat- polymerized acrylic resin (mainly MMA) (lot no.10NOV087)	Heraeus Kulzer, Sanden, Germany
	Methyl formate	Methyl formate (lot no. s246689)	Merck Schuchardt OHG, Germany
	Methyl acetate	Methyl acetate (lot no.s6328911)	Merck Schuchardt OHG, Germany

After deflasking, the specimens (Fig. 1a) were sectioned longitudinally and perpendicular to the bonding interface to prepare 2 mm thick test specimens using a low speed cutting machine (Isomet 1000, Buehler, Illinois, USA) (Fig. 1b). Rectangular specimens (4X11 mm) were prepared with a carbide bur and placed in a metal jig (Fig. 1c). A steel fissure bur was used in a surveyor (Fig. 1d) to prepare the specimens to create small dumbbell-shaped specimens with a 2.5x2 mm bond area (Fig. 1e). The dumbbell shape was similar to the model of Nakabayashi *et al*[20], but smaller in size (Fig. 2).

The specimens were stored in distilled water at 37°C for 24 hours. The specimens were attached to a metal holder and were fixed with screws (Fig. 3). Tensile bond strength testing was performed using a testing machine (EZ-S, Shimadzu, Kyoto, Japan) at a crosshead speed of 1 mm/min. The tensile bond strength was calculated by dividing the failure force by the adhesion surface area.

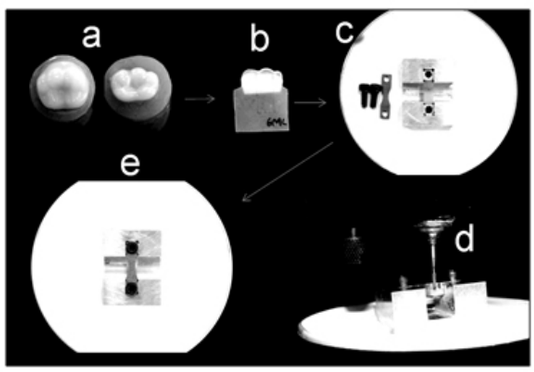

Figure 1 *Diagram of specimen preparation (a). the specimen after deflasking (b). 2 mm thick test specimens after cutting by low speed cutting machine (c). Rectangular specimen was placed in a metal jig for preparing dumb bell-shaped specimens (d).a steel fissure bur in a surveyor (e). dumbbell-shaped specimens*

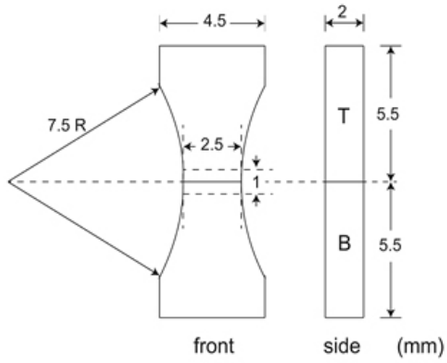

Figure 2 *Illustration of small dumbbell shaped specimen. T = denture teeth, B = denture base*

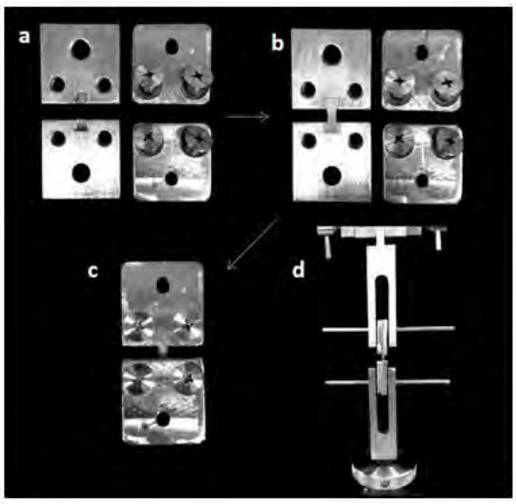

Figure 3 *Diagram of specimen fixation with metal holder and screws. (a) A metal holder and screws (b) A dumbbell-shaped specimen were placed in the metal holder (c) A dumbbell-shaped specimen were attached in the holder (d) A metal holder were attached with the universal testing machine*

To determine if the treated or untreated surfaces underwent any morphological changes, several denture teeth from each group were sputter-coated with gold and observed under a scanning electron microscope (JEOL-5410, JEOL Inc., Tokyo, Japan) at 15 kV. The fractured surfaces were examined using a stereo microscope (ML9300, Meiji Techno, Saitama, Japan) at 15X magnification and scanning electron microscopy (JEOL-5410, JEOL Inc., Tokyo, Japan) to determine the mode of failure. In the present study, the fractured surface was categorized as mixed primarily adhesive when the majority of the fracture was at the interface. If the majority of fracture occurred in the denture base, the specimen was categorized as having mixed primarily cohesive in denture base type failure. If a fracture occurred entirely in the denture base, the specimen was categorized as having cohesive failure of the denture base. There were no pure adhesive fractures because the SEM micrographs showed that in every fractured specimen, small fragments of denture base could be seen in the denture teeth.

The data were statistically analyzed using SPSS for Windows 17.0 (SPSS Inc., Chicago, IL, USA). The results were tested to determine the normality of distribution with the One-sample Kolmogorov-Smirnov test and the homogeneity of variance using the Levene's test. The data were normally distributed ($p>0.05$) and presented homogeneous variances ($p>0.05$), which indicated that a parametric analysis should be performed. The means and standard deviations for the tensile bond strength were calculated and statistically analyzed using two-way ANOVA and one-way ANOVA post hoc Tukey ($\alpha=0.05$). The modes of failure were analyzed using the Chi-square test.

Results

The results showed that surface treatment affected the bond strength between the denture teeth and the denture base. The surface treated groups demonstrated higher tensile bond strengths than the untreated groups ($p<0.05$), except for the 70:30 % v/v group (Table 2). Overall, the Major dent teeth groups exhibited significantly higher bond strength compared to the Cosmo HXL groups ($p<0.05$). Within each surface treatment group, there was no significant difference between the bond strength of conventional denture teeth and highly cross-linked denture teeth ($p>0.05$)

The mode of failure analysis (Table 3) revealed that the mode of failure was independent of the type of denture teeth and surface treatment ($p>0.05$). The negative control groups exhibited 100 % mixed, primarily adhesive failures. Chemical treated denture teeth with MMA or MF-MA resulted in an approximately 20 % decrease in the adhesive fracture (approximately 13 % mixed, primarily cohesive in denture base failure, and 8 % cohesive failure in denture base). Notably, Cosmo

HXL teeth treated with MF-MA at ratios of 40/60 or 70/30 exhibited a 40 % decrease in mixed, primarily adhesive failures (3/1 mixed, primarily cohesive in denture base failure/cohesive failure in denture base).

Table 2 *Mean and standard deviation (SD) of the tensile bond strength between denture teeth and denture base*

Chemical surface treatment	Denture teeth	
	Major dent	Cosmo HXL
Control	41.18 ± 6.12 [c, d]	37.51 ± 5.84 [d]
monomer (MMA)	56.10 ± 6.69 [a]	51.78 ± 6.39 [a, b]
MF 25 MA 75	56.66 ± 5.49 [a]	52.96 ± 7.51 [a, b]
MF 40 MA 60	53.50 ± 2.43 [a]	49.30 ± 6.46 [a, b, c]
MF 55 MA 45	55.79 ± 3.79 [a]	50.68 ± 6.87 [a,b]
MF 70 MA 30	49.25 ± 6.48 [a, b, c]	44.39 ± 4.62 [b, c, d]

MF = methyl formate, MA = methyl acetate, MMA = methyl methacrylate

**There was no significant difference (P>0.05) between groups denoted by the same letter.*

Table 3 *Mode of failure (n = 10 in each group).*

Chemical surface treatment	Denture teeth					
	Major dent			Cosmo HXL		
	mixed primarily adhesive	mixed primarily cohesive in denture base	cohesive in denture base	mixed primarily adhesive	mixed primarily cohesive in denture base	cohesive in denture base
Control	10	0	0	10	0	0
monomer (MMA)	9	0	1	9	0	1
MF 25 MA 75	8	1	1	9	1	0
MF 40 MA 60	7	1	2	6	3	1
MF 55 MA 45	8	1	1	8	2	0
MF 70 MA 30	9	1	0	6	3	1
Total	**51**	**4**	**5**	**48**	**9**	**3**

The SEM images of the untreated denture teeth showed a homogeneous surface with irregularities from grinding (Fig.4A, 5A). Treatment with acrylic denture base liquid created a blended and smoother surface (Fig.4B, 5B). Treatment with MF-MA solutions created surface pores (Fig.4C-F, 5C-F).

The top panel of Figure 6 shows an example of a mixed primarily cohesive in denture base failure specimen. The lower left panel shows the fracture at the interface area with a small fragment of the denture base attached to the denture tooth. The lower right panel shows the fracture in the denture base.

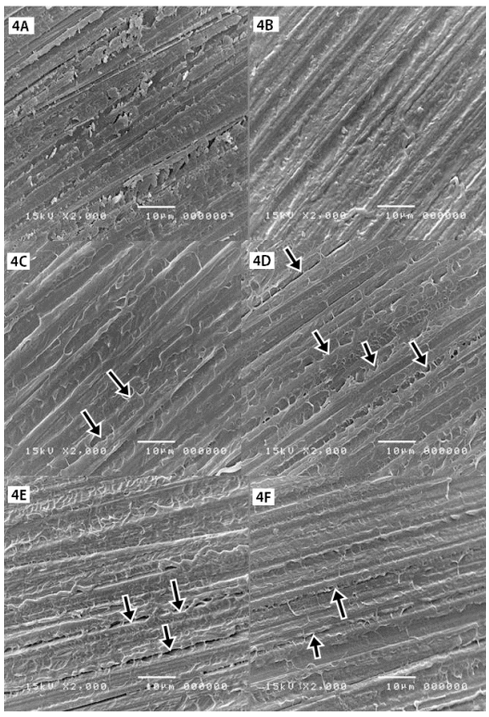

Figure 4 *SEM micrographs of the morphology of the untreated and treated surfaces of the Major dent denture teeth at 2000x magnificent level. (A) untreated (B) treated with MMA for 15 seconds. (C) treated with MF-MA solution (25:75 v/v). (D) treated with MF-MA solution (40:60 v/v). (E) treated with MF-MA solution (55:45 v/v). (F) treated with MF-MA solution (70:30 v/v). Black arrows indicate the pores on treated teeth surface*

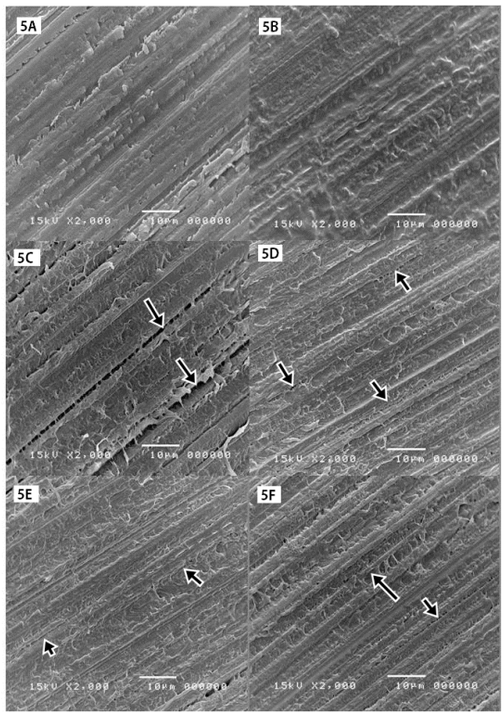

Figure 5 *SEM micrographs of the morphology of the untreated and treated surfaces of the Cosmo HXL denture teeth at 2000x magnificent level. (A) untreated (B) treated with MMA for 15 seconds (C) treated with MF-MA solution (25:75 v/v) (D) treated with MF-MA solution (40:60 v/v) (E) treated with MF-MA solution (55:45 v/v) (F) treated with MF-MA solution (70:30 v/v). Black arrows indicate the pores on treated teeth surface.*

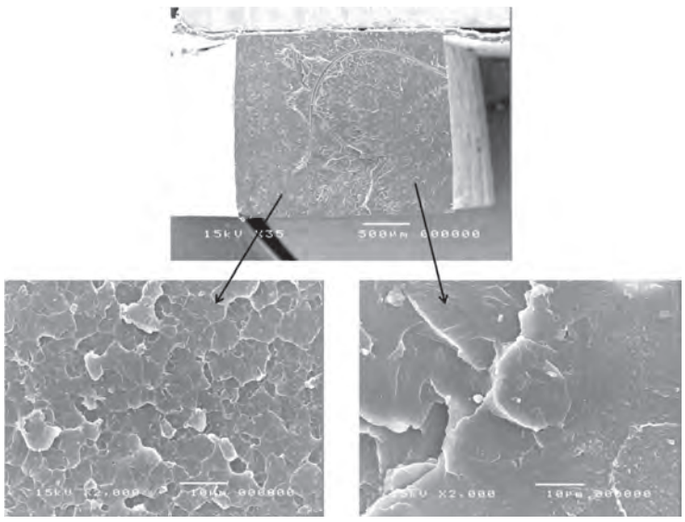

Figure 6 *SEM micrographs of a denture tooth after fracture at 35x magnificent level. The left arrow shows the fracture at the interface at 2000x magnificent level, the right arrow shows the fracture in denture base at 2000x magnificent level. This specimen was categorized as mixed primarily cohesive in denture base.*

Discussion

There are wide variations in the testing methods used for determining the bond strength between denture teeth and denture base.[21] ADA No.15[22] uses a tensile test but does not specify the bond area, while ISO 22112:2005[23] is only concerned with the mode of fracture. The present study used a tensile test and dumbbell shaped specimens according to the standard test for plastics (JIS K-691124, ISO527-125). The advantage of this design is that the tensile load was applied directly to the smallest part of the specimen, which was at the tooth-denture base interface.[20] A cross-section of 2.5x2.0 mm was prepared to maintain the correct dumbbell shape proportions based on the size of the denture teeth.

Based on these results, the first null hypothesis of surface treatment was rejected. The chemical surface treatments, used in this study, enhanced the bond strength between acrylic denture teeth and denture base. The swelling phenomenon occurs in acrylic denture teeth[26] when the monomer from the denture base polymer diffuses into the acrylic resin teeth during the packing process. In the present study, the increase in bond strength may have been caused by the chemical solution dissolving and swelling the denture tooth surface. This facilitates the diffusion of monomer from the denture base resin to form an interpenetrating polymer network with the denture teeth.

SEM images indicated that the denture teeth underwent morphological changes when treated with the chemical solutions. MMA created a blended and smoother surface on the denture teeth, while the MF-MA solutions created surface pores. These pores created space for the denture base resin and may have improved the bond strength due to micromechanical retention. However, there were no significant differences between the bond strengths of the MMA group and the MF-MA solution groups. This may result from the effect of the relatively high polymerization temperature, which can allow for greater monomer penetration.

Based on these results, the second null hypothesis of type of denture teeth was accepted. Conventional denture teeth demonstrated higher bond strength than the highly cross-linked denture teeth. However, there were no significant differences when compared by the same surface treatment. These results were confirmed by previous studies.[10,11] Conventional denture teeth have more unlinked polymer chains to develop an interwoven polymer network between the denture teeth and the denture base compared with the highly cross-linked denture teeth.[11] Differential scanning calorimetry has demonstrated that the glass transition temperature (T_g) of Major dent (115.1°C) is close to that of Cosmo HXL (117.8°C). The rate of monomer diffusion from the denture base resin is dependent on T_g.[27] This might be the reason why there was no significant difference in bond strength between conventional denture teeth and highly cross-linked denture teeth based on the surface treatment type. However, two-way ANOVA analysis indicated that the type of denture teeth had an effect on bond strength when comparing groups 1-6 with groups 7-12.

Vallitu[28] stated that an important factor for bond strength is that the bonding surface of the acrylic resin tooth must be adequately dissolved. In the present study, the period used for chemical agent exposure was 15 seconds. This application time was selected because it is practical for laboratory processing. The results of the chemical treated groups (except the 70:30 % v/v group) revealed that the 15 sec dissolving period was effective when compared with the control group.

According to the softening theory, a liquid will act as a plasticizer of a polymeric solid when the solubility parameters and polarities between the liquid and the polymeric solid are close to each other.[29] MMA, MA, and MF have solubility parameters of 18, 19.6, and 20.9 $MPa^{1/2}$ respectively.[30] These values approximate the solubility parameter of poly (methyl methacrylate) (18.3

$MPa^{1/2}$), which is the major component in acrylic denture teeth.

The bond strength of the 70:30 % v/v group was not significantly different from that of the untreated group. This can be attributed to the solubility parameters. The 70:30 % v/v group primarily contained MF that has a solubility parameter farther from that of poly (methyl methacrylate) compared with MA. Therefore, less dissolution and swelling of denture teeth surface likely occurred for the 70:30 % v/v group.

Table 4 show the toxicity and hazards of chemical solutions. NFPA (National Fire Protection Association) uses a standard called "NFPA 704" or "fire diamond" as a chemical hazard label.[31] There are four colored sections on the diamond. Number 0-4 are labelled in each section to indicate the level of hazard. On this scale, 0 indicates "no hazard" while 4 means "severe hazard". The blue section indicates health risk. The red section indicates flammability. Yellow indicates reactivity or explosivity. The white is section is used to describe any special hazards. The fire diamond indicated that the health risk of MMA[32] and MF[33] are level 2 which is greater than MA (level 1).[34] Level 2 means "Intense or continued non-chronic exposure may result in incapacitation or residual injury". Level 1 means "exposure may cause irritation and minor residual injury". Data from New Jersey Department of Health and Senior Services[35-37] indicated that the odor threshold of MF (2000 ppm) and MA (180 ppm) is more than MMA (0.049 ppm). In addition, data from OSHA, NIOSH, ACGIH indicated that the workplace airborne exposure limits of MMA is less or equal to MF and MA. These suggest that MF and MA are safer than MMA and can serve as a substitute to MMA as a surface treatment for denture teeth before packing acrylic resin in the dental laboratory.

Further research using a larger sample size, conditions similar to those found in the oral cavity, and a greater variation in application times is required to confirm the effect of MMA and MF-MA solution on the bond strength between acrylic denture teeth and a heated-polymerized acrylic denture base.

Table 4 *Toxicity and hazard of chemical solutions.*[32-37]

Materials	NFPA 704 or the Fire Diamond	Workplace exposure limit			
		Odor threshold	OSHA (8 hours)	NIOSH (10 hours)	ACGIH (8 hours)
Methyl methacrylate		0.049 ppm	100 ppm	100 ppm	50 ppm
Methyl formate		2000 ppm	100 ppm	100 ppm	100 ppm
Methyl acetate		180 ppm	200 ppm	200 ppm	200 ppm

ppm = parts per million *NIOSH = National Institute for Occupational Safety and Health*
OSHA = Occupational Safety and Health Administration *ACGIH = American Conference of Governmental Industrial Hygienists*

Conclusions

Within the limitations of the present study, the following conclusions can be drawn:

(1) The application of acrylic denture base liquid or a MF-MA solution (25:75, 40:60, 55:45 v/v) for 15 sec. to the base of denture teeth can increase the bond strength of acrylic denture teeth to the denture base.

(2) When comparing all the groups, the conventional denture teeth exhibited higher tensile bond strength than the highly cross-linked denture teeth. When comparing each surface treatment, the type of denture teeth had no effect on bond strength.

Acknowledgements

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References

1. Darbar UR, Huggett R, Harrison A. Denture fracture-a survey. ***Br Dent J*** 1994;176:342-5.

2. Cunningham JL. Bond strength of denture teeth to acrylic bases. ***J Dent*** 1993;21:274-80.

3. Fletcher AM, Al-Mulla MA, Amin WM, Dodd AW, Ritchie GM. A method of improving the bonding between artificial teeth and PMMA. ***J Dent*** 1985;13:102-8.

4. Cardash HS, Applebaum B, Baharav H, Liberman R. Effect of retention grooves on tooth-denture base bond. ***J Prosthet Dent*** 1990;64:492-6.

5. Chung KH, Chung CY, Chung CY, Chan DC. Effect of pre-processing surface treatments of acrylic teeth on bonding to the denture base. ***J Oral Rehabil*** 2008;35:268-75.

6. Sorensen SE, Fjeldstad E. Bonding of plastic to acrylic resin denture base materials. ***J Dent Res*** 1961;40:776.

7. Kurt M, Saraç YŞ, Ural C, Saraç D. Effect of pre-processing methods on bond strength between acrylic resin teeth and acrylic denture base resin. ***Gerodontology*** 2012;29:e357-62.

8. Barbosa DB, Monteiro DR, Barão VA, Pero AC, Compagnoni MA. Effect of monomer treatment and polymerisation methods on the bond strength of resin teeth to denture base material. ***Gerodontology*** 2009;26:225-31.

9. Saavedra G, Valandro LF, Leite FP, Amaral R, Ozcan M, Bottino MA, *et al.* Bond strength of acrylic teeth to denture base resin after various surface conditioning methods before and after thermocycling. ***Int J Prosthodont*** 2007;20:199-201.

10. Suzuki S, Sakoh M, Shiba A. Adhesive bonding of denture base resin to plastic denture teeth. ***J Biomed Mat Res*** 1990;24:1091-103.

11. Takahashi Y, Chai J, Takahashi T, Habu T. Bond strength of denture teeth to denture base resins. ***Int J Prosthodont*** 2000;13:59-65.

12. Korkmaz T, Dogan A, Dogan OM, Demir H. The bond strength of a highly cross-linked denture tooth to denture base polymers: a comparative study. ***J Adhes Dent*** 2011;13:85-92.

13. Meng GK, Chung KH, Fletcher-Stark ML, Zhang H. Effect of surface treatments and cyclic loading on the bond strength of acrylic resin denture teeth with autopolymerized repair acrylic resin. ***J Prosthet Dent*** 2010;103:245-52.

14. Lang R, Kolbeck C, Bergmann R, Handel G, Rosentritt M. Bond of acrylic teeth to different denture base resins after various surface-conditioning methods. ***Clin Oral Investig*** 2012;16:319-23.

15. Consani RL, Naoe HT, Mesquita MF, Sinhoreti MA, Mendes WB. Effect of ridge-lap surface treatments on the bond of resin teeth to denture base. ***J Adhes Dent*** 2011;13:287-93.

16. Civjan S, Huget EF, de Simon LB. Modifications of the fluid resin technique. ***J Am Dent Assoc*** 1972;85:109-12.

17. Morrow RM, Matvias FM, Windeler AS, Fuchs RJ. Bonding of plastic teeth to two heat-curing denture base resins. ***J Prosthet Dent*** 1978;39:565-8.

18. Thunyakitpisal N, Thunyakitpisal P, Wiwatwarapan C. The effect of chemical surface treatments on the flexural strength of repaired acrylic denture base resin. ***J Prosthodont*** 2011;20:195-9.

19. Osathananda R, Wiwatwarrapan C. Surface treatment with methyl formate-methyl acetate increased the shear bond strength between reline-resins and denture base resin. ***Gerodontology*** 2016;33:147-54.

20. Nakabayashi N, Watanabe A, Arao T. A tensile test to facilitate identification of defects in dentine bonded specimens. ***J Dent*** 1998;26:379-85.

21. Patil SB, Naveen BH, Patil NP. Bonding acrylic teeth to acrylic resin denture bases: a review. ***Gerodontology*** 2006;23:131-9.

22. American National Standards Institute. Revised ANSI/ADA specification no. 15. for Synthetic Resin Teeth. New York: ANSI/ADA; 1985.

23. International Organization for Standardization. ISO/TR 22112. Dentistry - Artificial teeth for dental prostheses. Geneva:ISO; 2005.

24. Japanese Industrial Standard. JIS K-6911 Testing methods for

thermosetting plastics. Tokyo:JIS: 1994.
25. International Organization for Standardization. ISO/TR 527-1 Plastics – Determination of tensile properties. Geneva:ISO; 1993.
26. Vallittu PK, Ruyter IE, Nat R. The swelling phenomenon of acrylic resin polymer teeth at the interface with denture base polymers. ***J Prosthet Dent*** 1997;78:194-9.
27. Sperling LH. Introduction to the Physical Polymer Science. New York: Wiley, 1986. p. 97-119.
28. Vallittu PK. Bonding of resin teeth to the polymethyl methacrylate denture base material. ***Acta Odontol Scand*** 1995;53:99-104.
29. Asmussen E, Peutzfeldt A. Substitutes for methylene chloride as dental softening agent. ***Eur J Oral Sci*** 2000;108:335-40.
30. Grulke EA. Solubility parameter values. In: Brandrup J, Immergut EH, Grulke EA, eds. Polymer Handbook 4th ed. New York: Wiley, 1999. p. 696-97, 708.
31. NFPA 704 or the Fire Diamond. Available at: http://chemistry.about.com/od/safetysignsandsymbols/a/Nfpa-704-Or-The-Fire-Diamond.htm (cited 2018 Apr 23)
32. Sciencelab.com, Inc.: Material Safety Data Sheet of Methyl methacrylate. Available at:www.sciencelab.com/msds.php?msdsId=9927360 (cited 2018 Apr 23)
33. Sciencelab.com, Inc.: Material Safety Data Sheet of Methyl formate. Available at:www.sciencelab.com/msds.php?msdsId=9926061 (cited 2018 Apr 23)
34. Sciencelab.com, Inc.: Material Safety Data Sheet of Methyl acetate. Available at:www.sciencelab.com/msds.php? msdsId=9927568 (cited 2018 Apr 23)
35. New Jersey Department of Health and Senior Services: Hazardous Substance Fact Sheet: Methyl Methacrylate. Available at: http://nj.gov/health/eoh/rtkweb/documents/fs/1277.pdf (cited 2018 Apr 23)
36. New Jersey Department of Health and Senior Services: Hazardous Substance Fact Sheet: Methyl Formate. Available at: http://nj.gov/health/eoh/rtkweb/documents/fs/1262.pdf (cited 2018 Apr 23)
37. New Jersey Department of Health and Senior Services: Hazardous Substance Fact Sheet: Methyl Acetate. Available at: http://nj.gov/health/eoh/rtkweb/documents/fs/1217.pdf (cited 2018 Apr 23)

Original Article

Micro-computerized tomographic Evaluation of Reparative Dentin Formation after Direct Pulp Capping *In vivo*

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Abstract

Dental pulp vitality can be preserved after direct pulp capping with materials that can promote hard tissue formation. The comparison of efficacy regarding dentin bridge formation of different direct pulp capping materials is still limited. The objective of this study was to analyze and quantify the reparative dentin formation after direct pulp capping with different direct pulp capping materials on mechanically injured dental pulp of rat molars through micro-computerized tomographic analysis. Fifteen rat molars were mechanically exposed and assigned into three groups according to the direct pulp capping materials used: no treatment (control), calcium hydroxide (Dycal®) and Biodentine. After four weeks of treatment, the teeth were collected and processed for micro-computerized tomographic imaging and histological evaluation. The ratio between total dentin volume and tissue volume was evaluated using micro-computerized tomographic analysis software. Hematoxylin and eosin staining of the samples treated with calcium hydroxide and Biodentine revealed the hard tissue formation resembled reparative dentin around the exposure site where the capping materials were placed. Micro-computerized tomographic imaging identified the location of reparative dentin formation after direct pulp capping. The average ratio of total dentin volume and tissue volume was 0.6055±0.0641 in the control group, 0.7381±0.0535 in the Biodentine group and 0.7099±0.0361 in the calcium hydroxide group. The calcium hydroxide and Biodentine® treated groups had a significantly higher total of dentin volume and tissue volume ratio compared with the untreated control group (p=0.008). However, no statistical significance was observed between the calcium hydroxide and the Biodentine® treated groups. Calcium hydroxide and Biodentine® can promote reparative dentin formation when used as direct pulp capping materials. In addition, micro-computerized tomographic imaging can be considered as a standard technique to quantify and localize the location of reparative dentin formation.

Keywords: Biodentine, Direct pulp capping, Reparative dentin, Micro-CT, Vital pulp therapy

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Introduction

Vital pulp therapy is a treatment to preserve the tooth vitality of teeth destroyed by dental caries, trauma, or restorative procedures. This technique is important especially for young permanent teeth with incomplete root development.[1] Direct pulp capping is one of the vital pulp therapy techniques. It is employed for pinpoint mechanical exposure of the dental pulp.[2] The capping material is placed in contact with the exposed pulp tissue in order to promote pulp tissue healing and subsequently induces the formation of reparative dentin to prevent bacterial invasion and to preserve dental pulp vitality.[3]

There are many materials that are used for direct pulp capping. Calcium hydroxide[4-6] is generally used as pulp capping material however Biodentine has recently been introduced as the candidate material of choice.[7] Clinical studies have shown several aspects of success with these materials such as the absence of symptoms or pathological results.[8] In both human and animal studies, histological evaluation of pulp capping materials assessed the inflammatory response of pulp and revealed that dentin bridge formation was stimulated underneath the material.[5,7,9]

Although the histologic evaluation of reparative dentin formation after direct pulp capping is commonly accepted as the gold standard, micro-computerized tomographic imaging is a noninvasive technique that can preserve dental hard tissue after investigation.[10] Hence, this study aimed to analyze and quantify the reparative dentin formation after direct pulp capping on mechanically injured dental pulp of rat molars through micro-computerized tomographic analysis.

Materials and Methods

Rat Molar Mechanical Pulp Injury Model

The animal experiment was approved by the Ethical Committee of the Faculty of Dentistry, Chulalongkorn University (Ethic NO.1732001). Eight 8-week-old, male Wistar albino rats *(Rattus norvegicus)* were obtained from the National Laboratory Animal Center, Mahidol University. Rats were given general anesthesia with sodium pentobarbital (40 mg/kg body weight) by intraperitoneal injection. Then, local anesthesia was applied at the surgical site with 2 % lidocaine with epinephrine 1:100,000 (4.4 mg/kg/dose). Intentional mechanical injury was performed by drilling on the mesial surface of first maxillary molars until near exposure, followed by creating a mechanical exposure with a sharp instrument and treated with three different pulp capping agents implanted into the exposure site using a blunt steel probe. The teeth were assigned into three groups: no treatment (control), calcium hydroxide (Dycal®, Dentsply, USA) or Biodentine (Septodont, France). Thereafter, the cavities were sealed with glass ionomer restorative materials (Fuji II LC®, GC, Tokyo). Four weeks after surgery, the animals were euthanized by sodium pentobarbital via intraperitoneal (120 mg/kg body weight) delivery. The use of sodium pentobarbital was modeled from the study of Limjeerajarus *et al*[11], and approved from the Ethical Committee of the Faculty of Dentistry, Chulalongkorn University to be a non-suffering method for rats. The whole maxilla of rats was sectioned and fixed in 10 % buffered formalin for 48 hours.

Micro-computerized tomography analysis

The whole maxilla was separated to the right and the left side. Hard tissue formation was evaluated using micro-computerized tomography (μCT35, Scanco Medical, Switzerland). The specimens were scanned with the following parameters: 70 kVp, 114 μA, 8 watts, and voxel size 10 μm. The same threshold was assigned to all sample analyses in the study. The analyses were performed using software from Scanco Medical. Each sample was analyzed with 60 slides in horizontal plane that the exposure involved. The area of pulp was selected

by the inner wall of dentin (Fig 1h). The ratio of total dentin volume and tissue volume was evaluated.

Histological analysis

Specimens were decalcified and embedded in paraffin blocks. The sections of two microns in thickness were obtained and stained with hematoxylin & eosin for histological evaluation. Each sample was investigated for tissue reaction by a blind investigator. The location and severity of inflammatory reaction, the presence and quality of dentin bridge formation were evaluated.

Statistical analysis

Results of the micro-computerized tomography analysis were expressed as mean±standard deviation. The Kruskal-Wallis test was performed to determine significant difference. Then, the Mann-Whitney test was run between the control and the experimental groups. All data was analyzed to determine the significant difference between the samples using IBM SPSS software version 22.0 (IBM, Armonk, NY, USA). Results were considered statistically significant at $p<0.05$.

Results

Hard tissue formation occurred underneath pulp capping material four weeks after the pulp capping procedure. Micro-computerized tomographic imaging revealed hard tissue formation at the exposure site and the total dentin volume/tissue volume ratio at the level of the mechanical pulp exposure of the experimental groups were higher than those of the control group (Fig. 2). The average ratio of total dentin volume/tissue volume was 0.6055±0.0641 in the control group, 0.7381±0.0535 in the Biodentine treated group and 0.7099±0.0361 in the calcium hydroxide treated group. The calcium hydroxide and Biodentine treated groups exhibited significantly higher total dentin volume/tissue volume ratio than the untreated control ($p=0.008$). Moreover, total dentin volume/tissue volume ratio of the Biodentine treated group was higher than the calcium hydroxide group. However, there was no statistically significant difference ($p=0.421$). A three-dimensional model of the defects is also demonstrated in Figure 1g.

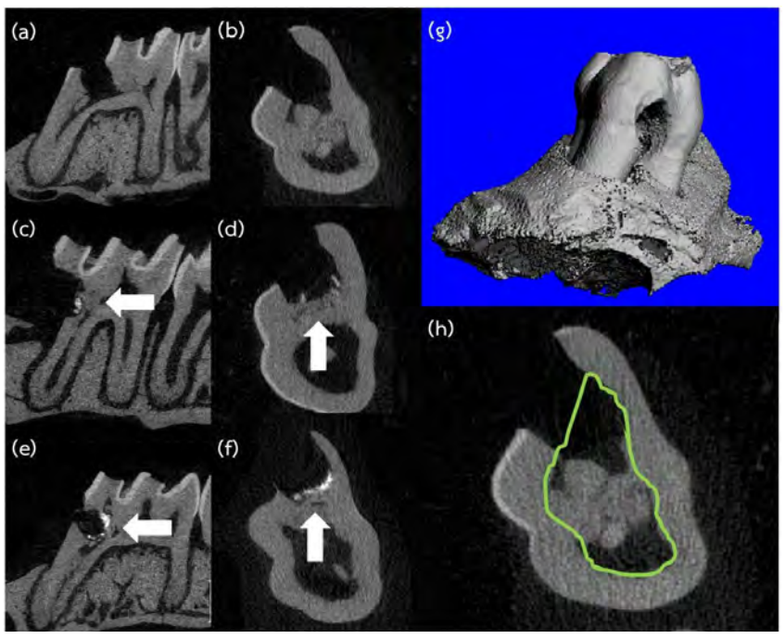

Figure 1 *Micro-computerized tomography images which show hard tissue formation occurring underneath pulp capping material after pulp capping procedures: the untreated control (a,b), calcium hydroxide (c,d), and Biodentine (e,f). Representative image of three-dimensional model of the defects (g). The area was selected to calculate the ratio of total dentin volume/tissue volume (h).*

Histological staining of reparative dentin formation showed the additional information of pulpal response after direct pulp capping (Fig. 3). The untreated group showed only fibrous tissue formation near the exposure site and abundant inflammatory response. Inflammatory cell infiltration involved more than 75 % of coronal pulp tissue. There was no reparative dentin formation in the control group. On the contrary, the calcium hydroxide and the Biodentine treated groups stimulated bridge formation with mineralized reparative dentin. Reparative dentin was seen at the exposure site and adjacent to former dentin close to the exposure site. Moreover, pulp tissue underneath the reparative dentin formation was similar to normal pulp tissue. There was minimal inflammation in the group treated with calcium hydroxide or Biodentine. Also, there were less blood vessel dilation and inflammatiry cell infiltration than in the control group.

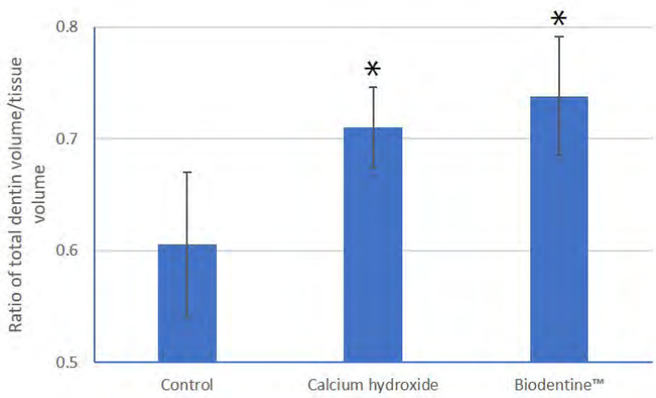

Figure 2 *Graph demonstrates the ratio of total dentin volume/tissue volume. The calcium hydroxide and Biodentine® treated group had a significantly higher ratio of total dentin volume/tissue volume than the control group. However, there was no significant difference between both pulp capping material treated groups. (* p<0.05).*

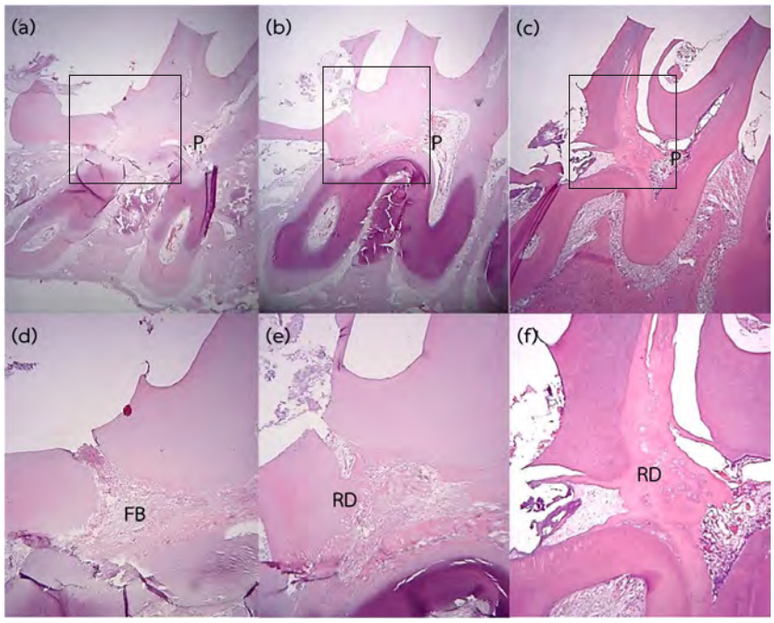

Figure 3 *Histological evaluation showed pulpal response after direct pulp capping: reparative dentin formation and inflammatory response (Magnification 4x (a-c) and 10x (d-e)). Reparative dentin formation (RD) occurred at exposure site and there was minimal inflammation after direct pulp capping with calcium hydroxide (b,e) or Biodentine (c,f). There were severe inflammation and reparation with fibrous tissue (FB) in the control group (a,d). (P; dental pulp).*

Discussion

Direct pulp capping is a treatment for vital pulp therapy. Pulp capping material should have proper properties for the healing process such as anti-bacterial, biocompatibility, hard tissue formation and tissue regeneration.[12-15] Presently, there are many materials used for this procedure. Calcium hydroxide paste or Dycal® and Biodentine are among the most popular material that dentists use in clinical practice. However, these materials are different in properties and results of treatment. It has been reported that both materials could promote dentin bridge formation after direct pulp capping.[13,16]

Micro-computerized tomography is one of the techniques that is employed to evaluate hard tissue formation without the destruction of the specimens. Ishimoto *et al* and Kim *et al* revealed that hard tissue formation was shown in micro-computerized tomography within four weeks after direct pulp capping.[10,17] Furthermore, the result of micro-computerized tomography was shown in three dimensions and the quantification of hard tissue was calculated with micro-computerized tomography software. In the present study, the total dentin volume/tissue volume ratio was analyzed based on the calculation of the volume of mineral tissue that is similar to hydroxyapatite and the total volume of interest. We selected the volume of interest that was involved in the mechanical exposure. It revealed hard tissue formation after mechanical exposure and the application of pulp capping materials in three dimensions. Hard tissue formation occurred after direct pulp capping. The use of biodentine and calcium hydroxide resulted in the formation of hard tissue with a significantly higher average of the total dentin volume/tissue volume ratio compared with the untreated group. However, the Biodentine group showed a higher average of the total dentin volume/tissue volume ratio than the calcium hydroxide group but there was no significant difference. According to Nowicka *et al,* the dentin bridge formation was evaluated with cone-beam computed tomographic (CBCT) imaging, the formation of bridge after direct pulp capping with calcium hydroxide had a similar result to Biodentine.[18]

While calcium hydroxide has antibacterial properties because of high pH, the result of calcium hydroxide is poor properties of dentin bridge formation. Calcium hydroxide can be resolved over time. Then, dentin bridge after direct pulp capping with calcium hydroxide revealed tunnel defect and osteodentin.[14,17,19-21] It consisted of porosity that can be a result of penetration of bacteria into the pulp cavity.[19] While, the dentin bridge formation of Biodentine had less porosity than calcium hydroxide.[17] Furthermore, the use of Biodentine showed a less inflammatory response in both the clinical[7] and animal studies.[22] Although many studies revealed that Biodentine stimulated reparative dentin formation and was a more preferable result than calcium hydroxide when used as pulp capping material. There were no previous studies done in the aspect of quantification by using micro-computerized tomography.

Although histological analysis is the most common investigation of reparative dentin formation after vital pulp therapy, it showed only two dimensions and could not showed the result in volume of reparative dentin formation. Moreover, it had technical sensitive because it required a specialist for interpretation. On the other hand, micro-computerized tomography showed the result in three dimensions and the specimens were not destroyed after investigation. The difference of former and reparative dentin could not be observed by using micro-computerized tomography. Therefore, histological staining was needed to confirm and identify reparative or former dentin.

Recently, the use of calcium hydroxide as pulp capping material has decreased. Despite being the gold standard for direct pulp capping in the past due to its antibacterial properties, calcium hydroxide exhibited

several drawbacks which can affect clinical outcomes. These disadvantages include pulpal inflammation and necrosis, microleakage from tunnel defects in the dentin bridge and degradation over the long term.[12,14] On the other hand, the dentin bridge completely formed after direct pulp capping with Biodentine and there was no inflammatory response in both the animal and clinical studies.[7,22]

Mechanism of reparative dentin formation after direct pulp capping occurred from the properties of the materials including high pH, antibacterial activity and calcium ion release.[23] Both calcium hydroxide and Biodentine have these properties. A high pH environment promoted osteogenic differentiation and bacterial irritation. Therefore, the process of reparative dentin formation was supposed to occur in this bacteria-free environment. Furthermore, calcium ions that were released from the materials was necessary for the formation of reparative dentin.

Mineral trioxide aggregate (MTA) is a pulp capping material. Because MTA is a calcium silicate based material in which the composition and properties is similar to Biodentine, many studies revealed favorable outcomes in both animal and clinical studies. From meta-analysis and a long-term study of MTA resulted in the decreased risk of failure and showed a significant higher success rate than calcium hydroxide.[24,25] High quality dentin bridge formation and less inflammation were observed after direct pulp capping with MTA.[26] Although calcium silicate base material revealed better results than the calcium hydroxide, MTA and Biodentine used in direct pulp capping procedures after caries removal in young permanent teeth showed a clinical outcome similar to calcium hydroxide.[27] Therefore, MTA is one of the suitable materials in vital pulp therapy. However, there has been no study done that studied the results of MTA using micro-computerized tomography. Therefore, micro-computerized tomographic evaluation of reparative dentin formation after direct pulp capping with MTA should be further studied.

Conclusion

Micro-computerized tomographic imaging can be considered as one of the standard techniques to quantify and localize the location of reparative dentin formation. Calcium hydroxide and Biodentine promote favorable results for direct pulp capping in terms of reparative dentin formation and reduction of inflammation.

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References

1. Ward J. Vital pulp therapy in cariously exposed permanent teeth and its limitations. ***Aust Endod J*** 2002;28:29-37.

2. American Academy of Pediatric Dentistry. Guideline on Pulp Therapy for Primary and Immature Permanent Teeth. ***Clinical Practice Guidelines*** 2015/16;37:244-52.

3. Murray PE, Hafez AA, Smith AJ, Cox CF. Bacterial microleakage and pulp inflammation associated with various restorative materials. ***Dent Mater*** 2002;18:470-8.

4. Caicedo R, Abbott PV, Alongi DJ, Alarcon MY. Clinical, radiographic and histological analysis of the effects of mineral trioxide aggregate used in direct pulp capping and pulpotomies of primary teeth. ***Aust Dent J*** 2006;51:297-305.

5. Min KS, Park HJ, Lee SK, Park SH, Hong CU, Kim HW, *et al.* Effect of mineral trioxide aggregate on dentin bridge formation and expression of dentin sialoprotein and heme oxygenase-1 in human dental pulp. ***J Endod*** 2008;34:666-70.

6. Tuna D, Olmez A. Clinical long-term evaluation of MTA as a direct pulp capping material in primary teeth. ***Int Endod J*** 2008; 41:273-8.

7. Nowicka A, Lipski M, Parafiniuk M, Sporniak-Tutak K, Lichota D, Kosierkiewicz A, *et al.* Response of human dental pulp capped with biodentine and mineral trioxide aggregate. ***J Endod*** 2013; 39:743-7.

8. Tran XV, Gorin C, Willig C, Baroukh B, Pellat B, Decup F, *et al.*

Effect of a calcium-silicate-based restorative cement on pulp repair. ***J Dent Res*** 2012;91:1166-71.

9. Accorinte ML, Loguercio AD, Reis A, Bauer JR, Grande RH, Murata SS, *et al.* Evaluation of two mineral trioxide aggregate compounds as pulp-capping agents in human teeth. ***Int Endod J*** 2009;42:122-8.

10. Kim J, Song YS, Min KS, Kim SH, Koh JT, Lee BN, *et al.* Evaluation of reparative dentin formation of ProRoot MTA, Biodentine and BioAggregate using micro-CT and immunohistochemistry. ***Restor Dent Endod*** 2016;41:29-36.

11. Limjeerajarus CN, Osathanon T, Manokawinchoke J, Pavasant P. Iloprost up-regulates vascular endothelial growth factor expression in human dental pulp cells *in vitro* and enhances pulpal blood flow *in vivo*. ***J Endod*** 2014;40:925-30.

12. Hilton TJ. Keys to clinical success with pulp capping: a review of the literature. ***Oper Dent*** 2009;34:615-25.

13. Ireland RL. Secondary dentin formation in the deciduous teeth. ***JADA*** 1941;28:1626-32.

14. Njeh A, Uzunoglu E, Ardila-Osorio H, Simon S, Berdal A, Kellrmann O, *et al.* Reactionary and reparative dentin formation after pulp capping: Hydrogel vs. Dycal. ***Evidence-Based Endodontics*** 2016;1:1-9.

15. Tziafas D. The future role of a molecular approach to pulp-dentin regeneration. ***Caries Res*** 2004;38:314-20.

16. Stanley HR. Criteria for standardizing and increasing credibility of direct pulp capping studies. ***Am J Dent*** 1998;11:S17-34.

17. Ishimoto K, Hayano S, Yanagita T, Kurosaka H, Kawanabe N, Itoh S, *et al.* Topical application of lithium chloride on the pulp induces dentin regeneration. ***PLoS One*** 2015;10:e0121938.

18. Nowicka A, Wilk G, Lipski M, Kolecki J, Buczkowska-Radlinska J. Tomographic evaluation of reparative dentin formation after direct pulp capping with Ca(OH)2, MTA, Biodentine, and dentin bonding system in human teeth. ***J Endod*** 2015;41:1234-40.

19. Cohenca N, Paranjpe A, Berg J. Vital pulp therapy. ***Dent Clin North Am*** 2013;57:59-73.

20. Nair PN, Duncan HF, Pitt Ford TR, Luder HU. Histological, ultrastructural and quantitative investigations on the response of healthy human pulps to experimental capping with Mineral Trioxide Aggregate: a randomized controlled trial. 2008. ***Int Endod J*** 2009;42:422-44.

21. Suzuki M, Taira Y, Kato C, Shinkai K, Katoh Y. Histological evaluation of direct pulp capping of rat pulp with experimentally developed low-viscosity adhesives containing reparative dentin-promoting agents. ***J Dent*** 2016;44:27-36.

22. Shayegan A, Jurysta C, Atash R, Petein M, Abbeele AV. Biodentine used as a pulp-capping agent in primary pig teeth. ***Pediatr Dent*** 2012;34:e202-8.

23. Song M, Yu B, Kim S, Hayashi M, Smith C, Sohn S, *et al.* Clinical and Molecular Perspectives of Reparative Dentin Formation: Lessons Learned from Pulp-Capping Materials and the Emerging Roles of Calcium. ***Dent Clin North Am*** 2017;61:93-110.

24. Li Z, Cao L, Fan M, Xu Q. Direct Pulp Capping with Calcium Hydroxide or Mineral Trioxide Aggregate: A Meta-analysis. ***J Endod*** 2015;41:1412-7.

25. Mente J, Hufnagel S, Leo M, Michel A, Gehrig H, Panagidis D, *et al.* Treatment outcome of mineral trioxide aggregate or calcium hydroxide direct pulp capping: long-term results. ***J Endod*** 2014;40:1746-51.

26. AlShwaimi E, Majeed A, Ali AA. Pulpal Responses to Direct Capping with Betamethasone/Gentamicin Cream and Mineral Trioxide Aggregate: Histologic and Micro-Computed Tomography Assessments. ***J Endod*** 2016;42:30-5.

27. Brizuela C, Ormeno A, Cabrera C, Cabezas R, Silva CI, Ramirez V, *et al.* Direct pulp capping with calcium hydroxide, Mineral trioxide aggregate, and Biodentine in permanent young teeth with caries: a randomized clinical trial. ***J Endod*** 2017;43:1776-80.

Original Articles

Different Cleansing Methods Effect to Bond Strength of Contaminated Zirconia

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Abstract

Saliva contamination on a restoration is unavoidable during a try-in procedure. Many studies have shown the negative effect of non-cleansing surface prior to cementation. The aim of this study is to investigate the efficiency of cleansing methods on the shear bond strength of zirconia surfaces. Sixty-six zirconia specimens size 7.5x5.5x0.8 mm were randomly divided into six groups: non-saliva contamination (PC), saliva contamination without surface cleansing (NC), saliva contamination then cleansing with Ivoclean (IC), 0.5 M NaOH solution (NaOH), sandblasting (SB) and sandblasting followed by 0.5 M NaOH solution (SB+NaOH). One specimen in each group was separated for SEM observation. The remaining zirconia specimens were bonded to a composite resin block with Panavia F2.0 and were stored in 37°C distilled water for 24 hours. All specimens were subjected to the SBS test. The data were analyzed by One-way ANOVA and Tukey HSD. The bonded surfaces were observed under stereomicroscope to identify the mode of failure. The results showed that the SBS of saliva contaminated zirconia without surface cleansing (NC) was the significantly lowest (4.62±0.53 MPa) than that of the other groups ($p<0.05$), while SB (14.14±1.72 MPa) and SB+NaOH (15.41±1.65 MPa) were significantly higher than the others ($p<0.05$). However, SB and SB+NaOH showed no statistically significant difference ($p>0.05$). Group PC, IC and NaOH showed no significant difference ($p>0.05$). The mode of failure revealed a greater amount of mixed failure for the majority of SB and SB+NaOH but other groups reveal adhesive failure between zirconia and resin cement for the majority. SEM showed surface morphology changing in SB and SB+NaOH when compared to other group. The saliva contaminated zirconia should be cleaned by Ivoclean, 0.5 M NaOH solution, sandblasting or sandblasting followed by 0.5 M NaOH solution prior to cementation.

Keywords: Bond strength, Saliva contamination, Sodium hydroxide, Surface cleansing, Zirconia

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Introduction

Nowadays, fixed partial restoration is one of the popular choices for restoring a destructed tooth. However, the longevity of the restoration may vary for each person because of many factors including caries, porcelain fracture, dislodgement of crown due to loss of retention, etc. The bonding between the restoration and the tooth structure is one of the most important factors that affect the longevity of the restoration. The bond strength may be affected by contamination on both the tooth and the restoration.

Contamination of saliva during the try-in procedure of restoration is unavoidable. Saliva has a negative effect on bonding which results in shorter longevity but could be solved by using various surface cleansing agents.[1] Many authors suggested the method of cleansing saliva-contaminated surfaces which could be classified into chemical and mechanical cleansing depending on the type of restoration.

The chemical cleansing could be done by using acid etching (i.e. phosphoric acid and hydrofluoric acid) and an alkaline-based agent (Ivoclean). Yang *et al,* 2008 found that phosphoric acid could recover the bond strength of a saliva-contaminated silica-based restoration but at the same time they were found that acid had a negative effect on decreasing the bond strength of a zirconia restoration. Hydrofluoric acid is one choice of surface cleansing of ceramic restoration, both silica-based and zirconia, which could recover bond strength after being saliva-contaminated. The mechanical cleansing could be done by sandblasting and tribochemical on a zirconia restoration. The sandblasting with 50-µm Al_2O_3 at 2.8 bars of pressure for 30 seconds at a distance of 10 mm could improve bond strength of saliva-contaminated zirconia.[2]

Recently, Ivoclean (an alkaline base surface cleansing agent) was introduced to clean the surface of a restoration before the bonding procedure. It desorbed phosphate, saliva phospholipid, from the surface of the restoration. The main composition of Ivoclean is sodium hydroxide (NaOH) solution which results in the improvement of bond strength between the restoration and the tooth structure. Furthermore, the zirconium oxide particles claimed to enhance the phosphate adsorption properties.[2-4] However, comparing the Ivoclean and non-commercial NaOH solution, non-containing zirconium oxide particles, on the shear bond strength of the saliva-contaminated zirconia restoration has not been investigated. The objective of this study is to compare the effect of different surface cleansing methods on shear bond strength of a zirconia restoration.

Materials and Methods

The sixty-six fully sintered zirconia specimens size 7.5x5.5x0.8 mm were prepared from a zirconia block (VITA YZ HT, VITA Zahnfabrik, Germany) and sintered followed the manufacturer's instruction. The specimens were randomly divided into six groups, ten specimens for shear bond strength (SBS) test and another specimen for Scanning electron microscope (SEM) observation (Quanta 250, Thermo Fisher Scientific, USA). The specimens were invested in the center of polyvinyl chloride (PVC) tube with a self-cured acrylic resin (Unifast Trad, GC corporation, Japan) by using a stainless-steel mold.

The 3 mm diameter with 4 mm height composite specimen (Tetric® N-Ceram shade A3.5, Ivoclar Vivadent, Switzerland) was fabricated by an incremental layering technique into the glass covered mold and were light-cured by a LED light curing unit (Elipar™, 3M ESPE, USA) with a light intensity of 1,200 mW/cm^2 for 20 seconds in each increment and an additional 20 seconds on every side before the specimens were removed from the mold. The sixty composite specimens were fabricated and randomly divided into six groups as zirconia specimens. The 0.5 M NaOH solution was prepared and the pH level of the solution was tested by a pH-Fix 0-14 test strip (Macherey-Nagel, Germany) to confirm that the pH

level was between 13–13.5. The solution was freshly prepared and kept in a covered volumetric flask.

The surfaces of the specimens were cleaned by immersion in 0.5 M NaOH solution for 8 hours then rinsed with deionized water for 20 seconds and dried with oil free air. The six groups of specimens were surface treated as shown in Figure 1. The sandblasting was operated by Al_2O_3 particles size 50 µm at distance of 10 mm under 0.2 MPa for 20 seconds.

The composite specimens were cemented to the zirconia specimen with Panavia F2.0 (Kuraray Dental, Japan). The cement was mixed by weighting paste A and paste B at about 0.02±0.001 mg using a four-point decimal digital scaler (Sartorius; Germany) as per manufacturer's instructions in mixing ratio of 1:1. The cement was applied on the zirconia surface and the composite specimens were placed under pressure of 1 kg for 8 minutes by a Durometer (ASTM D 2240 Type A, DPTC Instruments, USA). The excessive cement was gently cleaned and lightly cured for 20 seconds on each side following the manufacturer's instructions.

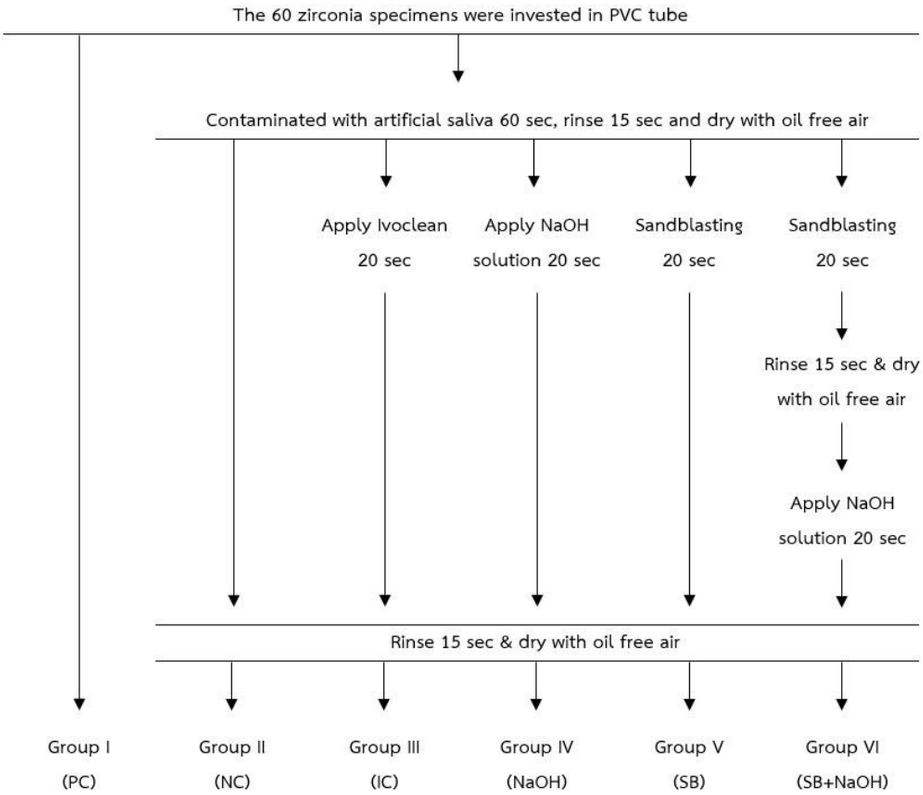

Figure 1 *Methods of surface treated in each group*

Then, the specimens were stored in 37°C distilled water for 24 hours in an incubator (Contherm 1200, New Zealand) before testing the shear bond strength. The shear bond strength test was conducted following the ISO 11405:2015 by Shimadzu universal testing machine (EZ-S, Shimadzu, Japan) with load cell 500 N at cross head speed of 1 mm/min until the bonded surfaces of the specimens were broken. The bonded surfaces of zirconia and the composite resin were investigated under stereomicroscope at 20x magnification to classify the mode of failure as adhesive, cohesive or mixed failure as the following:

Adhesive: The failure occurred between the interface of the zirconia specimen to the resin cement (Zr/P) or the resin cement to the composite specimen (P/Cr).

Cohesive: The failure occurred in the resin cement (P) or the composite specimen (Cr).

Mixed: The failure occurred in a combination of adhesive and cohesive that one of the failure occurred on more than 25 % of the bonded interface.

The six zirconia specimens were surface treated as the above methods. The surface morphologies were observed under SEM at 1000, 3000 and 5000x magnification.

The shear bond strength values were recorded and statistically analysis by One-way Analysis of Variance (ANOVA) and Tukey HSD at 95 % confidence level with SPSS 17 (SPSS Inc, Chicago, Illinois).

Results

Shear bond strength

The mean amount of shear bond strength was analyzed for normal distribution by the Komolgorov-Smirnov test and the data showed normal distribution in each group. Then, the data was analyzed by One-way ANOVA at 95 % confidence level. The results showed that there were significant differences between the groups. Thus, the null hypothesis was not accepted. The multiple comparisons of the shear bond strength were analyzed by Tukey HSD to identify the difference between the groups at 95 % confidence level.

As shown in Table 1, group NC showed the significantly lowest shear bond strength than the experimental groups ($p<0.05$). While group PC, IC and NaOH showed no statistically significant difference ($p>0.05$). Group SB and SB+NaOH showed statistically significant higher difference than the others ($p<0.05$). However, a comparison between group SB and SB+NaOH showed no statistically significant difference ($p>0.05$).

Table 1 *Means and standard deviations of the SBS of all of the tested groups in MPa (n = 10)*

Group	Mean ± SD (MPa)
PC	6.10 ± 0.62 ^{B}
NC	4.62 ± 0.53 ^{C}
IC	6.16 ± 0.62 ^{B}
NaOH	6.29 ± 0.80 ^{B}
SB	14.14 ± 1.72 ^{A}
SB+NaOH	15.41 ± 1.65 ^{A}

** The same superscript capital letter means there is no significant difference at 95 % confidence level.*

Mode of failure

The fractured interfacial zones on the zirconia and the composite specimens were examined under a stereomicroscope. The major failure of group PC, NC, IC and NaOH is adhesive failure between the zirconia specimens and the resin cement. The difference of group SB and SB+NaOH which had the major failure is mixed type (Table 2).

Table 2 *Mode of failure examined under a stereomicroscope.*

Group	Mode of failure (n)				
	Adhesive		Mixed	Cohesive	
	Zr/P	P/Cr		P	Cr
PC	7	0	3	0	0
NC	9	0	1	0	0
IC	9	0	1	0	0
NaOH	9	0	1	0	0
SB	0	1	9	0	0
SB+NaOH	0	0	10	0	0

Discussion

Surface cleansing methods on shear bond strength of saliva-contaminated zirconia

The number of specimens was calculated by power analysis of the pilot study and the results showed the ten specimens in each group is more than the calculated quantity.

Although the zirconia restoration is fabricated by CAD-CAM technique but the restoration still may not perfectly fit to the abutment and a try-in procedure is still necessary. Saliva contamination is unavoidable in the try-in procedure of the restoration. The saliva contamination on the surface of the zirconia restoration has shown significantly decreasing bond strength in several studies.[1,5] For this reason, the surface of the restoration should be cleaned prior to cementation. In this study, the shear bond strength of group NC has significantly decreased (p<0.05) when compared to a non-saliva contaminated surface (PC) and cleansing the saliva-contaminated surface with different methods; Ivoclean (IC), 0.5 M NaOH solution (NaOH), sandblasting (SB) and sandblasting followed by 0.5 M NaOH solution (SB+NaOH). This finding agreed with previous studies which showed that airborne particle abrasion and cleaning paste yielded higher bond strength value more than the other cleansing methods and airborne particle abrasion yielded the highest bond strength value.[2,5-7]

When the surface of the saliva-contaminated zirconia was cleaned with Ivoclean (IC) and 0.5M NaOH solution (NaOH) for 20 seconds, the shear bond strength was recovered and showed no significant difference to a non-contaminated surface (PC). This result agreed with previous studies that Ivoclean could clean contaminated-zirconia and recover equivalent or a greater bond strength as non-contaminated zirconia.[2,4,6] In this study, the 0.5 M NaOH solution with pH 13–13.5 was investigated due to several studies which found that NaOH solution can desorb phosphate and the amount of desorbed phosphate increased with the increase of the alkalinity of the NaOH solution. In contrast, the lower the pH, the phosphate could better absorb to the zirconia surface.[3,8]

In water pollution management, the zirconia beads are used in phosphate absorption from wastewater. The NaOH solution has been recommended for decontamination of the phosphate-contaminated zirconia before reusing. The phosphate desorption could be done by immersion of the zirconia beads in 0.1 M NaOH for 4 hours and 0.1 M NaOH for 12 hours which show the desorption rate at about 48.0 % and 91.7 % accordingly. The 0.5 M NaOH enhances the desorption of phosphate at more than 0.1 M NaOH but there was no significant difference.[8] However, in this study, immersion in NaOH solution for only 20 seconds could recover the same bond strength as non-contaminated zirconia.

The NaOH solution in this study is prepared to

a pH of about 13–13.5 which is the same as Ivoclean's conditions. However, the NaOH solution in this study is in the liquid form which is different from Ivoclean which is available as a gel. Gel is easier to use and could stick to the surface because of it's high viscosity. Thus, it was suggested that Ivoclean be used only 1 to 2 drops per time which is different from the liquid form that must be placed in a container such as beaker or small bowl. In the future, the NaOH solution in liquid form may made into a gel by adding glycerin. Moreover, it may also reduce tissue irritation from accidental contact or respiratory tract irritation from the evaporation of NaOH solution.

Sandblasting with 50 μm aluminum oxide particle at distance of 10 mm, pressure 0.2 MPa, for 20 seconds (SB) and sandblasting followed by the NaOH solution (SB+NaOH) shows a higher shear bond strength of saliva-contaminated zirconia than others. This may be caused from the surface characteristic after sandblasting which create more surface area when compared to a non-sandblasted surface. From the study of He *et al.,* the sandblasted surface of the restoration has more surface roughness than non-sandblasting of about 7.3 times and more than SBS at 1.3 times.[9]

Zirconia is acid-resistant but could be etchable under some specific conditions.[10] In a clinical situation, surface roughening of zirconia might be done by sandblasting which creates micromechanical retention to resin cement.[9] Sandblasting increases the surface area, surface energy, surface wettability and flowing ability of resin cement in to micro-retention areas. When the surface area increases, the oxide layer increases too. This causes more chemical bonding between the oxide layer to the MDP and may lead to higher bond strength. However, sandblasting may create surface flaws, reducing the strength of the restoration and accelerate tetragonal to monoclinic phase transformation.[9,11]

Comparing the sandblasting group (SB) with sandblasting followed by NaOH (SB+NaOH), there was no significant difference with the shear bond strength. The above results showed that sandblasting could remove the contamination on the zirconia surface due to the NaOH solution application after sandblasting could not improve any shear bond strength. The average shear bond strength in this study also showed a range of many previous studies, about 12-20 MPa.[4,12-14] This can conclude that only sandblasting with Al_2O_3 particles size 50 μm at distance of 10 mm, pressure 0.2 MPa for 20 seconds on saliva-contaminated zirconia could recover or yield higher the bond strength compared to non-contaminated zirconia.

Mode of failure

Sandblasting is the best way to improve bond strength and to clean the surface. The mode of failure of group SB and SB+NaOH are mostly mixed while shear bond strength show significant improvement over the others group that are mainly adhesive. This may result from sandblasting that roughens the surface which increases the surface area for retention of the crown by cementation.

The SEM showed that the surface morphology in groups PC, NC, IC, and NaOH were not different. Thus, after surface contamination and cleansing by using NaOH solution or Ivoclean did not affect the surface morphology. However, a more irregular surface was observed in group SB and SB+NaOH compared to the others (Fig. 2). This mode of failure from group SB and SB+NaOH has proven that bond strength is better after being cleaned and surface treated by sandblasting. Therefore, the zirconia restoration should be sandblasted before cementation for better retention.

In further studies, the surface of the zirconia should be investigated by Energy Dispersive Spectrometry (EDS) which can analyze the organic and inorganic elements to detect the amount of phosphate before/after contamination and after cleansing. Zirconia was chosen for investigation of zirconia-oxide which play an important role of chemical bonding to MDP. Thus, the non-precious metal restoration should be further investigated due to the surface of non-precious metal composed of metal-oxide which could bond chemically to MDP in the same way as zirconia.

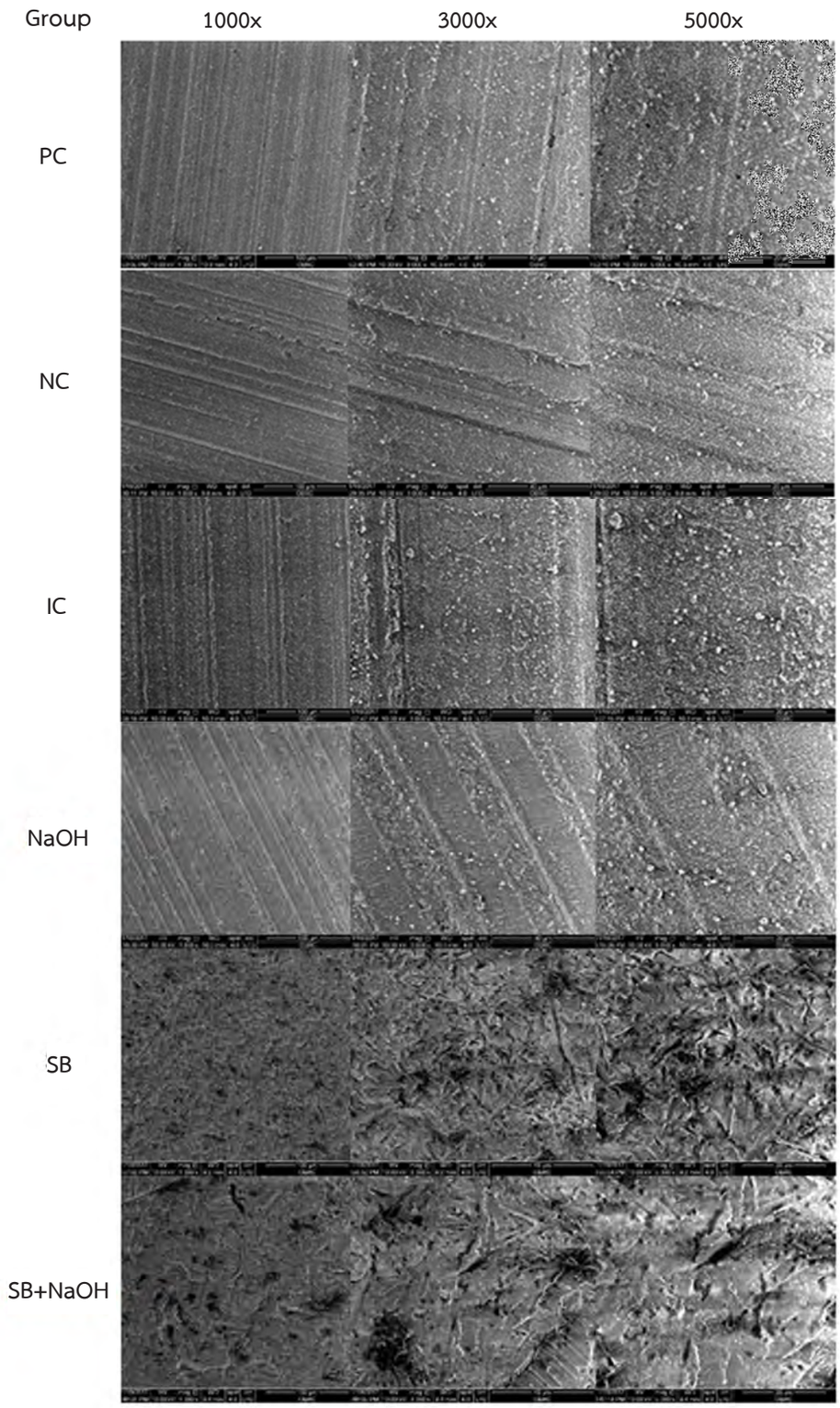

Figure 2 *SEM of zirconia specimens at 1000x, 3000x and 5000x magnificent level*

Conclusion

Saliva contamination significantly decreases the shear bond strength of a zirconia restoration. The recommended effective cleaning methods to remove saliva contamination and recover bond strength are following:

1. Chemical cleansing methods: Application of Ivoclean or 0.5 M NaOH solution for 20 seconds.

2. Mechanical cleansing methods: Sandblasting with Al_2O_3 particles size 50 μm at distance of 10 mm, pressure 0.2 MPa for 20 seconds.

Acknowledgments

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References

1. Luciana NG, Samuel NV, Tais S, Fernando AK, Maria APS, Adriana BM. Effect of saliva contamination on the bond strength of an etch-and-rinse adhesive system to dentin. ***Rev Odonto Cienc*** 2009;24:410-13.

2. Feitosa SA, Patel D, Borges ALS, Alshehri EZ, Bottino MA, Özcan M, *et al.* Effect of cleansing methods on saliva-contaminated zirconia—An Evaluation of Resin Bond Durability. ***Oper Dent*** 2015;40:163-71.

3. Su Y, Cui H, Li Q, Gao S, Shang JK. Strong adsorption of phosphate by amorphous zirconium oxide nanoparticles. ***Water Res*** 2013; 47:5018-26.

4. Kim DH, Son JS, Jeong SH, Kim YK, Kim KH, Kwon TY. Efficacy of various cleaning solutions on saliva-contaminated zirconia for improved resin bonding. ***J Adv Prosthodont*** 2015;7:85-92.

5. Yang B, Wolfart S, Scharnberg M, Ludwig K, Adelung R, Kern M. Influence of contamination on zirconia ceramic Bonding. ***J Dent Res*** 2007;86:749-53.

6. Tunc EP, Chebib N, Sen D, Zandparsa R. Effectiveness of different surface cleaning methods on the shear bond strength of resin cement to contaminated zirconia: an *in vitro* study. ***J Adhes Sci Technol*** 2016;30:554-65.

7. Yang B, Lange-Jansen HC, Scharnberg M, Wolfart S, Ludwig K, Adelung R, *et al.* Influence of saliva contamination on zirconia ceramic bonding. ***Dent Mater*** 2008;24:508-13.

8. Ren Z, Shao L, Zhang G. Adsorption of Phosphate from aqueous solution using an Iron–Zirconium Binary Oxide sorbent. ***Water Air Soil Pollut*** 2012;223:4221-31.

9. He M, Zhang Z, Zheng D, Ding N, Liu Y. Effect of sandblasting on surface roughness of zirconia-based ceramics and shear bond strength of veneering porcelain. ***Dent Mater J*** 2014;33:778-85.

10. Sriamporn T, Thamrongananskul N, Busabok C, Poolthong S, Uo M, Tagami J. Dental zirconia can be etched by hydrofluoric acid. ***Dent Mater J*** 2014;33:79-85

11. Tanış MÇ, Akçaboy C. Effects of different surface treatment methods and MDP monomer on resin cementation of zirconia ceramics an *In vitro* study. ***J Lasers Med Sci*** 2015;6:174-81.

12. Della Bona A, Borba M, Benetti P, Cecchetti D. Effect of surface treatments on the bond strength of a zirconia-reinforced ceramic to composite resin. ***Braz Oral Res*** 2007;21:10-5.

13. May LG, Passos SP, Capelli DB, Ozcan M, Bottino MA, Valandro LF. Effect of silica coating combined to a MDP-based primer on the resin bond to Y-TZP ceramic. ***J Biomed Mater Res B Appl Biomater*** 2010;95:69-74.

14. Aladag A, Elter B, Comlekoglu E, Kanat B, Sonugelen M, Kesercioglu A, *et al.* Effect of different cleaning regimens on the adhesion of resin to saliva-contaminated ceramics. ***J Prosthodont*** 2015;24:136-45.

Original Article

Characteristics of Adipose-derived Stem Cells Isolated from Buccal Fat Pads Using CD 271 Cell Sorting Technique

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Abstract

Buccal fat pad is a suitable intra-oral source of Adipose-Derived Stem Cells (ADSCs) for bone tissue engineering. CD271 is one of the most specific cell surface markers used to isolate mesenchymal stem cells from various tissues. However, there has not been a study done that has used the cell-sorting technique with this marker to isolate the ASC from buccal fat tissue. The aim of this study was to compare the characteristics of ADSCs isolated from intraoral buccal fat pads using CD 271+ magnetic-activated cell sorting (MACS) and plastic adherence (PA). Buccal fat tissue was harvested from ten patients who underwent orthognathic surgeries. ADSCs were isolated from the tissue using PA (Group A) and MACS; CD 271+ (Group B) and CD271- (Group C), (5 participants per group). The characteristics of the cells including colony forming unit fibroblast (CFU-F), immunophenotyping markers, and multi-differentiation into tri-lineages were comparatively assessed. Gingival fibroblast served as the negative control group. The results demonstrated that (CFU-F) formed in the Group B cells, but were not detected in the other groups. The cells of groups A-C expressed the mesenchymal stem cell including CD 73, 90 and 105. No statistical difference was detected among the groups. It was noted that CD 73 was detected at the highest levels followed by CD 105 and CD 90 respectively. The cells of the control group expressed those markers remarkably less than the experiment groups (significant differences were found in CD 73 and CD 105, $p<0.05$). In addition, the cells of all groups expressed hematopoietic stem cell markers including CD 14, 20, 34 and 45 at very low levels. The cells of groups A-C demonstrated adipogenic, chondrogenic and osteogenic differentiation when cultured in the inductive conditions. There was no significant difference of those properties among the groups. In conclusion, CD 271 is considered as a proper marker for sorting ADSCs from buccal fat tissue. However, it cannot be used as the sole marker. Although the ADSCs expressed CD 90 at the lowest levels, they still had osteogenic differentiation capacity. Therefore, they can be used as a stem cell source to repair bone defects.

Keywords: Buccal fat stem cells, Adipose-derived stem cells, Stromal vascular fraction, Mesenchymal stem cell, CD271, surface markers, cell sorting technique

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Introduction

Tissue engineering has become a popular alternative method in the field of reconstructive surgery. The bone tissue engineering triad includes scaffolds, cells, and signaling substances. A combination of scaffolds with bone forming cells as cell-scaffold constructs is a good strategy to enhance bone regeneration. Several studies[1-5] obtained better results in promoting new bone when the scaffolds were combined with several cell types, such as primary osteoblasts, mesenchymal stem cells (MSCs) from bone marrow (BM) (BM-MSCs), and dental pulp. Another source of MSCs is fat tissue from which adipose-derived stem cells (ADSCs) are obtained. ADSCs were found to express immunophenotyping markers similar to the BM-MSCs. Moreover, they can be differentiated toward various cell types, especially bone forming cells. Some studies[6-10] revealed that the buccal fat pads are suitable intra-oral sources of the ADSCs which provide a large amount of fat tissue that is easily harvested in routine intraoral surgical fields. Plastic adherence (PA) capacity of stem cells is commonly used to isolate the ADSCs from other cell types since non-adherent cells can be washed out after periods of culture. Although this technique is very simple and cheap, the amount of stem cells obtained is only about one cell per 105 of adherent cells.[11,12] A new method to purify stem cell populations is magnetic-activated cell sorting (MACS) which uses antibody-binding. The specific markers are conjugated to iron oxide microbeads that can retain the desired cells in the column containers in a magnetic field, whereas unlabeled cells are eluted.[13] Some studies[14-17] suggest using low-affinity nerve growth factor receptor (CD271,p75NTR) antibody as a positive selection marker of MSCs. The CD271+ cells obtain high purity of a MSC population that supports self-renewal capacity and multi-differentiation potential. However, there are no comparative results of characterization of the ADSCs isolated from the intraoral buccal fat pads using the conventional PA method or MACS. In this study, CD271 was used as the specific marker of the MSCs and the positive and negative cells to the marker were included for the investigation.

Materials and Methods

Patient enrollment

Ten volunteer patients were enrolled in the study. All patients underwent orthognathic surgeries to correct skeletal discrepancies at the Oral & Maxillofacial Surgery Clinic, Dental Hospital, Faculty of Dentistry, Prince of Songkla University. The protocol of this study was approved by the ethics committee of the Faculty of Dentistry, Prince of Songkla University (EC5909-38-P-LR). The inclusion criteria of the participants included ASA class I, age >20 years old, weight >50 kg and hematocrit ≥35 %. The excluded patients were those with systemic diseases including hereditary blood diseases, disorders of the blood and blood components, blood transmitted diseases, and diabetes.

Isolating ADSCs from fat tissue

The ADSCs of the participants were consecutively isolated using two different methods and divided into three groups. In Group A, five samples of cells were isolated from five patients using PA.[18] The other five patients were the sources of cells for Group B (five samples) and Group C (five samples). In Group B, the cells which were positive to CD 271 (CD271+) were isolated using MACS. In Group C, the cells which were negative to CD271 (CD271-) were isolated using MACS. Each participant underwent orthognathic surgery under general anesthesia. During the operations of Lefort I osteotomy of the maxilla or sagittal split osteotomy of the mandible, some parts of the fat pads were excised, then immediately placed into DMEM (Dulbecco's Modified Eagle Medium, Gibco, USA) and stored at 4°C until the isolation process. The fat tissue was washed several times with sterile phosphate buffered saline (PBS) to remove contaminating debris and red blood cells. The volumes were then measured using a 5 mL sterile disposable syringe. Afterwards, the tissues were minced into small

pieces and enzymatically digested using 0.75 % collagenase type I (Gibco, USA) in PBS at 37°C with gentle agitation for 60 min. The supernatants were collected, and then centrifuged at 400 g for 10 min to exclude the remaining adipocytes and lipid droplets. The cellular pellets were suspended in DMEM supplemented with 10 % fetal bovine serum (FBS) (Gibco, USA), and then filtered through a 100 µm filter (Corning, USA). In group A the cell suspension was plated onto 6-well culture plates (Corning, USA) and cultured in a humidified atmosphere with 5 % CO_2 at 37°C. In groups B and C, the cells were isolated using CD271 antibody for MACS (CD271 MicroBead Kit human, Miltenyi Biotec, Germany). In brief, the cell suspension was re-centrifuged at 300 g for 10 min and the cell pellets were resuspended in 60 µL of buffer (PBS with 0.5 % FBS, and 2 mM EDTA). The cell suspensions were incubated in 20 µL of FcR blocking reagent and 20 µL of CD271 MicroBeads for 15 min at 4°C. Afterwards, the cells were washed by adding 1-2 mL of buffer, and then centrifuged at 300 × g for 10 min. Re-suspended cell solutions were made by adding 500 µL of buffer, and then loaded onto a column which was placed in a MiniMACS separator (Miltenyi Biotec, Germany). The magnetically labeled CD271+ cells of group B were retained in the column, whereas the unlabeled CD 271- cells of group C were run through. The cells of Groups B and C were collected and cultured in DMEM supplemented with 10 % FBS in a humidified atmosphere with 5 % CO_2 at 37°C. The cells of all groups at passages 1-4 were used for the following experiments (five samples/group/test).

Determining the Characteristics of the cells

Colony-forming unit fibroblast (CFU-F) assays

The cells of each group at passage 1 were plated at a density of 100 cells/well in a 6-well plate to define the numbers of CFU-F. Within 20 days, the cells were fixed with 4 % paraformaldehyde and stained with 0.1 % toluidine blue (Sigma, USA). The CFU-F was observed under a light microscope (Nikon, Japan). The numbers of colonies were counted if the aggregations were at least 50 cells or the colonies were >2 mm in diameters.[2] The cells of each patient were assessed in triplicate.

Flow cytometry analysis

The MSC immunophenotypes of the ADSCs were defined following the International Society for Cellular Therapy (ISCT) protocols.[19] The analysis was performed using a fluorochrome-conjugated monoclonal antibody cocktail in the MSC Phenotyping Kit human (Miltenyi Biotec, Germany). In brief, 5 x 10^5 cells from each group in passages 2 and 3 were incubated in the antibodies against the surface antigens CD73, CD90, and CD105 as the positive markers and CD14, CD20, CD34, and CD45 as the negative markers. At least 10,000 events were acquired for each sample using a fluorescent-activated cell sorting instrument (FACSCalibur, BD Biosciences) and the data were analyzed using CELLQUEST software (BD Biosciences).

Multi-differentiate potential of the ADSCs

Adipogenic differentiation

The cells at 1 x 10^4/well were cultured in adipogenic induction medium comprised of DMEM supplemented with 10 % FBS, 1 µM dexamethasone, 10 µg/mL insulin, 500 µM 3-isobutyl-1-methylxanthine and 200 µM indomethacin (Sigma, USA) for 21 days. The culture mediums were changed every two days. On day 21, the cells were fixed in 10 % formaldehyde for 1 h and stained with Oil Red O solution (20 mg/mL in isopropanol) (Sigma, USA) for 15 min. Lipid vacuoles were quantified by extracting with 100 % isopropanol for 10 min and reading with a microplate reader (Multiscan™Go, Thermo Fisher Scientific) at the absorbance of 540 nm.

Chondrogenic differentiation

The cells (5 x 10^5) were centrifuged at 600 g for 5 min to form cell pellets and re-suspended in 2 mL chrondogenic medium (StemPro Chondrogenesis Differentiation Kit, Gibco) in a 15-mL centrifugation tube (Corning, USA) for 21 days to induce chondrogenic differentiation.[20,21] The culture medium was changed every three days.[20] Determination of expression of

chondrogenic differentiation was performed after 21 days of culture using alcian blue staining.[20]

Osteogenic differentiation

The cells (1 x 10^4 cells/well) were cultured in osteogenic medium (DMEM supplemented with 10 % FBS, 5 mM beta-glycerophosphate, 100 nM dexamethasone, and 50 µg/mL ascorbic acid). Osteoblastic differentiation markers including alkaline phosphatase activity (ALP) and osteocalcin expression (OCN) at days 3, 7, 14, and 21 were assessed using ELISA (five samples/group/time point). On the days of the experiments, the cells were washed two times using PBS. After that, 200 µL of 1 % Triton X-100 in PBS was added into each well and then the cells were lysed by freezing and thawing in three cycles (30 min/cycle). The mixtures were transferred into microcentrifuge tubes and centrifuged at 2000 x g for 10 min. The supernatants were collected as cell lysis solutions and kept at -80°C for the analysis of total cellular protein content, ALP activity, and OCN expression. The quantification of total protein in the solutions were performed according to the manufacturer's instructions (Bio-Rad Protein Assay; Bio-Rad Laboratories, USA) based on the method of Bradford. Absorbance at 750 nm was read using the microplate reader. The ALP activities were measured according to the instructions using the commercial kit of Alkaline Phosphatase, AMP Buffer (HUMAN, Germany) according to the recommendation of the International Federation of Clinical Chemistry. The levels of activity were calculated per one milligram of the total cellular protein (U/L/mg protein). Quantification of OCN was performed according to the manufacturer instructions using the Osteocalcin ELISA kit (Biomedical Technologies Inc., USA). The solutions were read at 450 nm absorbance using the microplate reader and their concentrations were calculated with the serial diluted standard solution. The OCN levels were demonstrated as ng/mg protein.[22]

Statistical Analysis

The measured parameters were analyzed using statistics analysis software (SPSS, version 22.0, USA). One-way analysis of variance (ANOVA) and Tukey HSD were applied to compare the differences among the groups and time points. The level of statistical significance was set at $P<0.05$.

Results

The average volume of buccal fat tissue harvested from the patients was 3.9 ± 2.6 mL.

CFU-F

CFU-Fs were detected only in Group B during 20 days of culture, whereas CFU-Fs were not detected in Groups A or C (Fig. 1).

Flow cytometry analysis

Expression of the MSC immunophenotypes of the ADSCs is demonstrated in Figure 2 and Table 1. Among the groups, the profiles of the positive markers of MSC were not statistically different. The cells of all groups expressed CD73 at the highest levels, followed by CD105 and CD90. Expression of the hematopoietic markers of all groups was less than 1 %. The fibroblasts expressed CD73 and 105 significantly less than those of Groups A and B ($P<0.05$).

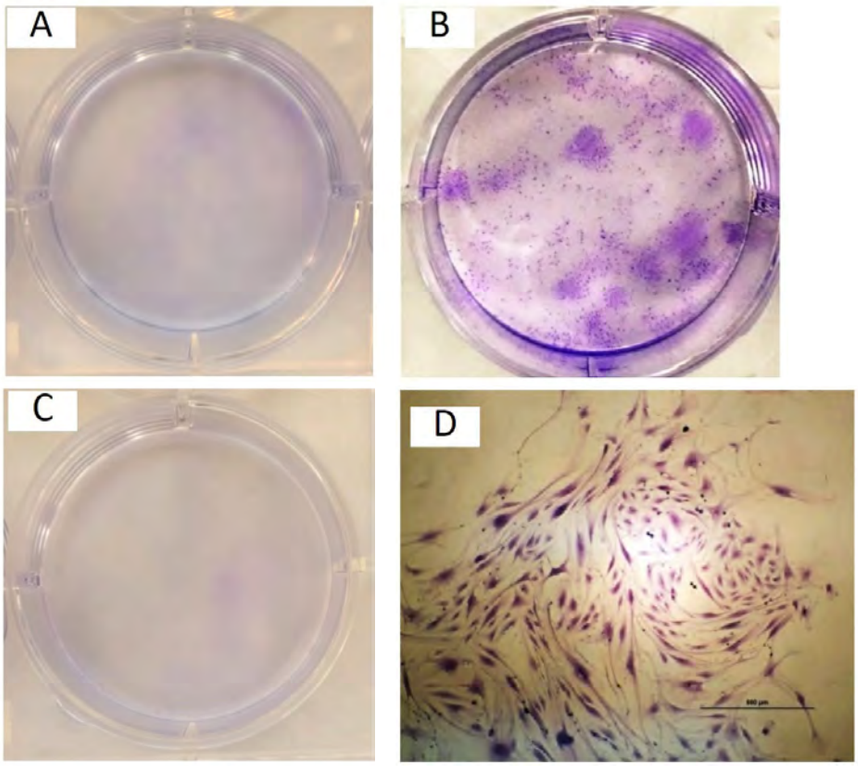

Figure 1 *Toluidine blue staining of CFU-F after 20 days of culture. A: Group A, B: Group B and C: Group C. D: The magnified image of Group B CFU-F.*

Table 1 *The percentages of immunophenotyping markers were demonstrated.*

CD Markers (%)	Groups	A (PA)	B (CD271+)	C (CD271-)	Gingival Fibroblasts (control)
MSCs markers	CD 90	54.5±27.4	48.7±16.7	58.1±12.6	56.4±9.3
	CD 105	78.7±12.1	60.6±9.7****	62.7±11.7*****	44.2±13.7##
	CD 73	88.8±5.3*	89.9±6.3**	86.7±13.5***	33.9±5.4#
Hematopoietic markers	CD 14, 20, 34, 45	0.71±0.4	0.72±0.4	0.99±0.1	0.2±0.17

*The percentages of CD73 of groups A-C were significantly higher than CD90 (*p=0.03, **p=0.002, ***p=0.006). The percentages of CD105 of groups B and C were significantly higher than CD90*
*(****p=0.018, *****p=0.02). The percentages of CD90, CD105 and CD73 of the control group were not significantly different. CD 73 and CD 105 of this group were significantly less than those of group A and B (#p =0.002, ##p=0.007))*

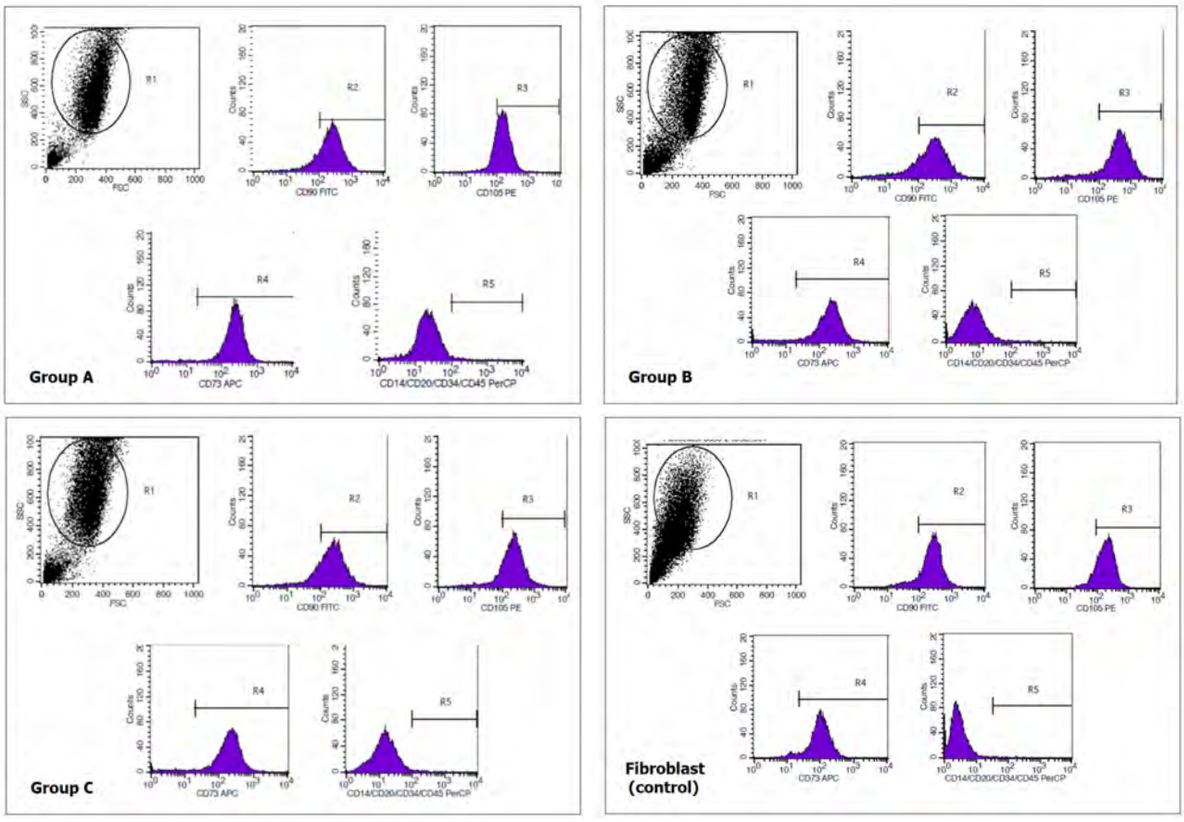

Figure 2 *The pictures of flow cytometry analysis show the profiles of the MSC markers and the hematopoietic markers of the experiment groups.*

Multi-differentiate potential of the ADSCs

Adipogenic differentiation

After 21 days of culture, lipid vacuoles were detected in red (Fig. 3A). The quantitative measurement of the extracted lipid vacuoles is demonstrated in Figure 3B. There was no statistical difference among the groups ($P<0.05$).

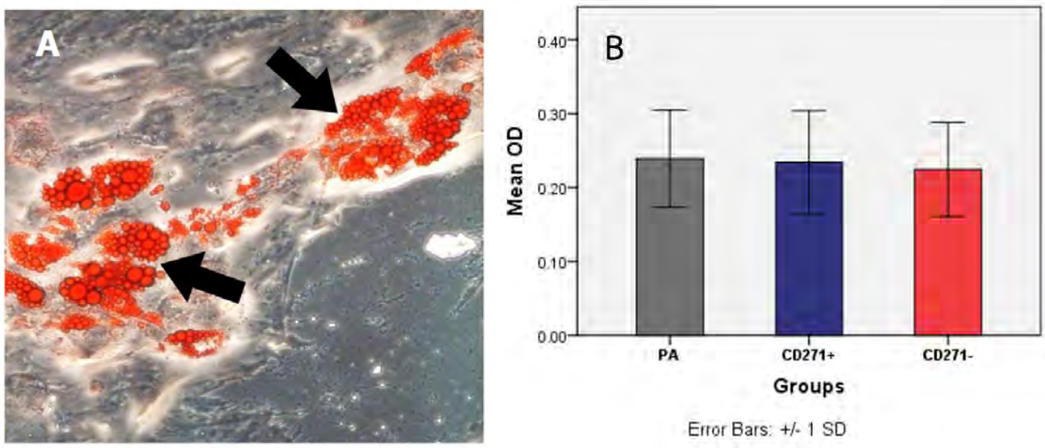

Figure 3 *(A) Oil Red O staining demonstrates the lipid vacuoles seen in red (arrows). (B) The bar graphs demonstrate the OD levels of the solubilized Oil Red O. No significant differences were detected among Groups A-C.*

Chondrogenic differentiation

After inductive culture, the cell pellets of Groups A-C could produce cartilaginous matrix (Fig. 4).

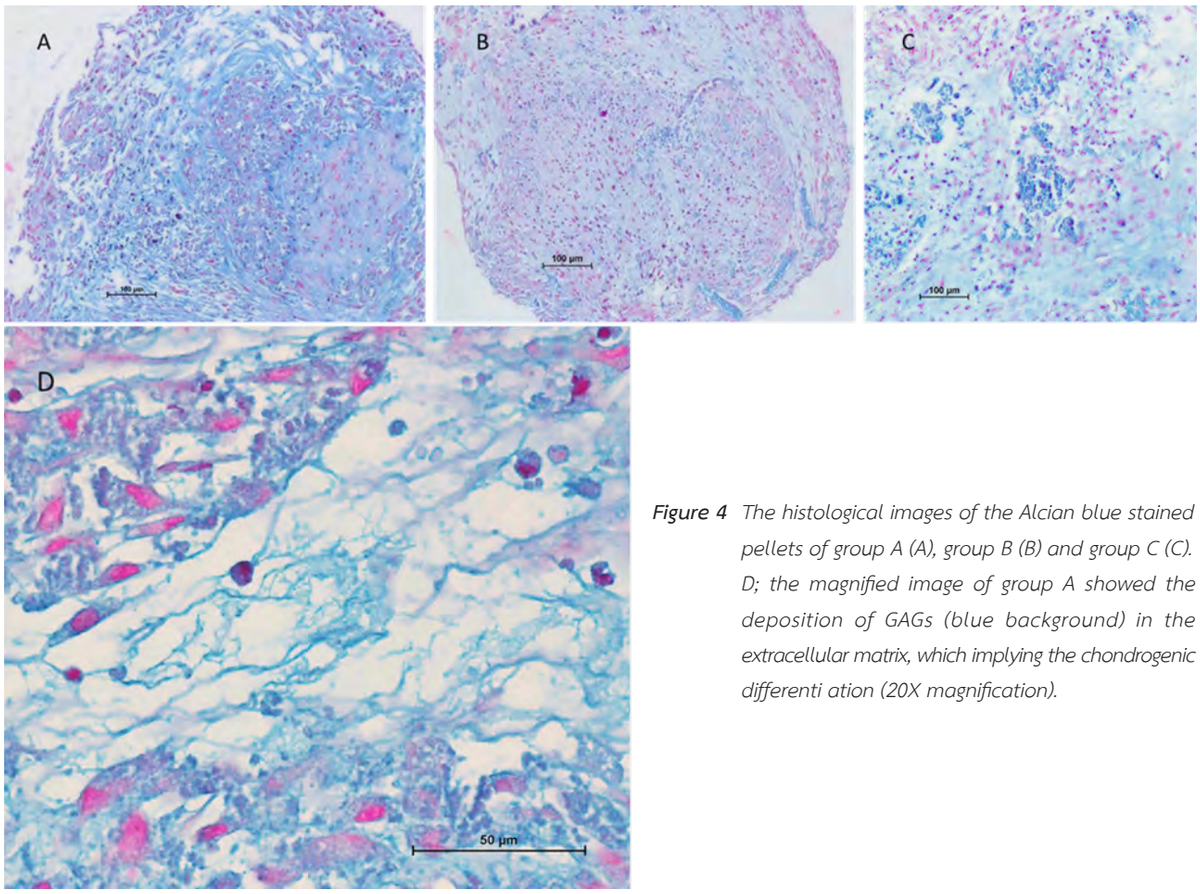

Figure 4 *The histological images of the Alcian blue stained pellets of group A (A), group B (B) and group C (C). D; the magnified image of group A showed the deposition of GAGs (blue background) in the extracellular matrix, which implying the chondrogenic differenti ation (20X magnification).*

Osteogenic differentiation

The ALP levels of Groups A-C are demonstrated in Figure 5. The levels of ALP of Groups A and B seemed to be stable during the first 14 days, and then they noticeably increased at day 21. On day 21, the ALP level of Group A was significantly greater than the other groups (P=0.000). In Group C, the highest ALP was detected at day 3 and then the levels decreased on the following days. The OCN levels are shown in Figure 6. The levels of OCN in Groups A and B rapidly increased to reach the highest levels on day 7, and then decreased thereafter. The highest expression of OCN in Group C was detected at day 3, and then the levels rapidly decreased on the following days.

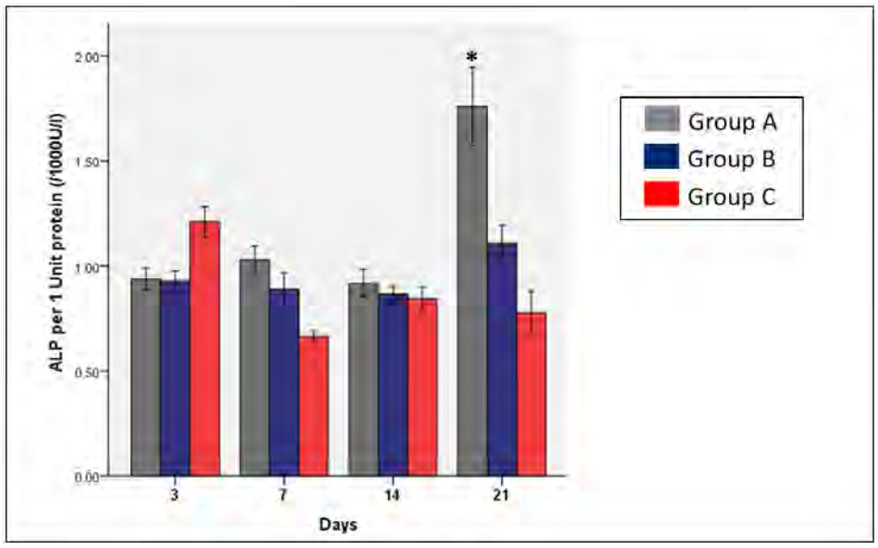

Figure 5 *The bar graphs demonstrate ALP activities of group A-C. The data showed that there was no statistically difference of the ALP levels among the groups on the first 14 days of culture. On day 21, the level of group A was significantly greater than the other groups (*p=0.001).*

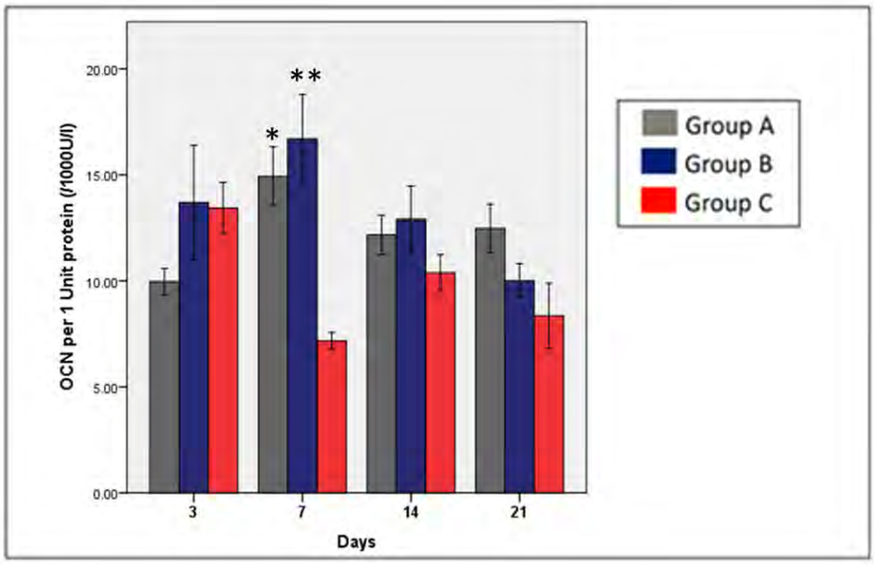

Figure 6 *Bar graphs demonstrate the OCN levels of Groups A-C. The maximum levels of Groups A and B were detected on day 7, whereas the maximum level was detected in Group C on day 3. The osteocalcin levels of Groups A and B were significantly higher than group C (*p=0.004,**p=0.001 respectively).*

Discussion

In the field of bone tissue engineering, adipose tissue is an alternative potential source of mesenchymal stem cells which have the ability to differentiate toward the lineage of osteoprogenitor cells.[6,23,24] Buccal fat pads are excellent intra-oral sources of adipose tissue that provide greater volumes of tissue compared with dental pulp and periodontal ligament. Moreover, the harvesting technique of the tissue is easily performed under local anesthesia which is less invasive compared with bone marrow sources. Plastic adherence is a conventional

technique used to isolate stem cells from tissue. The technique is routine and easy, but after enzymatic digestion, adipose tissue generates a pellet of stromal vascular fraction (SVF) which contains a heterogeneous cell population. At the cellular level, SVF is composed of mature adipocytes, fibroblasts, nerve cells, endothelial cells, immune cells, and preadipocytic cells.[25,26] Those cells usually have various protein and cytokine expressions and potency of differentiation.[25] Therefore, methods to purify them are still interesting. Cell sorting with specific surface markers to isolate the cells can obtain a more homogenous cell population. MACS would be the optimum method for identification of stem cells in clinical practices due to the fact that it can be done as a chairside procedure and the isolation processes can be finished within two hours. Moreover, it remarkably reduces cultivation time, and avoids contamination of the cell culture reagents, when compared with the conventional plastic adherence method. However, the amounts of stem cells from buccal fat tissue, retained in the column of MACS might be low and they should be further assessed. Several markers have been used to isolate MSCs from various sources. Nevertheless, a standard acceptable definition has not reached a consensus. The two types of CD surface markers of stem cells are sole markers and stemness markers. A sole marker is considered to be sufficient to identify stem cells from their in vivo environment, whereas stemness markers are used to identify subsets of cells with high CFU-Fs and trilineage potential. In principle, the sole markers are highly expressed, while the stemness markers may be moderately detected.[27] CD271 is considered to be one of the most specific markers to isolate MSCs from bone marrow, dental pulp, and adipose tissue.[14-16,28-32] A recent study[31] stated that CD271 is the best single marker to isolate dental pulp mesenchymal stem cells with the greatest differentiation potential. However, there has not been a study done which has used this marker to isolate ADSCs from buccal fat pads. Our study is the first to demonstrate the characteristics of the CD 271+ ADSCs isolated from buccal fat pads using the cell sorting method in terms of expression of MSC markers and the capacity to exhibit trilineage differentiation. Their properties were compared with those isolated with conventional plastic adherence and the gingival fibroblasts.

The results of CFU-F assay revealed that self-renewal capacity of the cells was detected only in the CD 271+ cells, whereas that property was not detected in the PA cells or in the CD 271- cells. These results corresponded with some previous studies.[14,15,29,33] Poloni, *et al.* compared numbers of CFU-Fs generated by human BMSCs isolated using Ficoll gradient and CD271+ mononuclear cells isolated using MACS. After 14 days of culture, the authors found higher numbers of CFU-Fs of the CD271+ cells compared with the unsorted BM-MSCs[15] Quirici, *et al.* determined the clonogenic potential in three different populations from human adipose tissue including PA, CD271+ cells, and CD34+ cells. At less than 10 weeks of culture, there were no detectable significant differences in the numbers of CFU-Fs among the groups. However, after 20 weeks, the number of CFU-Fs in the PA group nearly disappeared which was significantly less than in the CD34+ and 271+ groups.[29] Kuçi, *et al.* demonstrated that CFU-F activity was found only in the CD271+ cells, while there were no CFU-Fs detected in the CD271– cells.[14] Jarocha, *et al.*[33] compared the capacity for CFU-Fs among various methods of isolating MSCs from bone marrow including PA, RosetteSep-isolation, and CD105+ and CD271+ selection. The results showed that the CD271+ fraction had the highest number of CFU-F colonies compared with the other groups.[33] It is possible that the heterogeneous cells isolated using PA and the CD271- cells were possibly contaminated with hematopoietic cells, endothelial cells, erythrocytes, fibroblasts, lymphocytes, monocytes, and macrophages. The strong proliferation of cells can diminish the growth of progenitor cells.[34,35] Therefore, selective isolation using a specific marker can promote better self-renewal capacity.

Based on the minimum criteria of ISCT, the cells

that display the properties of PA, positive expression of CD90, CD105, CD73, and negative expression of hematopoietic markers, and multi-differentiation potency can be termed "MSC".[36] Our results demonstrated that the averages cell populations of PA, CD271+, and CD271- groups positively expressed CD90, CD105, and CD73 were less than 90 %, while the negatively expressed hematopoietic markers of CD14, CD20, CD34, and CD45 were less than 1 % without a significant difference among the groups. The positive cell numbers were less than the ISCT criteria to identify MSC, which purposes that the cell population should express the CD73, CD90 and CD105 ≥ 95 %.[36] However, our results corresponded to the consensus between ISCT and International Federation for Adipose Therapeutics and Science (IFATS)[35], which purposes that the ASC should be positive to CD13, CD29, CD44, CD73, CD90, and CD105 (>80 %) and negative to CD31, CD45, and CD235a (<2 %). Whereas, the SVF should express the primary markers of stromal cells including CD13, CD29, CD44, CD73, CD90 (>40 %), and CD34 (>20 %), but express the negative markers of CD31 (<20 %) and CD45 (<50 %).

Interestingly, the control group of fibroblasts could express the MSC markers, but the amounts were remarkably less than the experiment groups (significant differences were found in CD 73 and CD 105, $P<0.05$). In addition, they negatively expressed the hematopoietic markers less than the other groups. This character corresponded to previous studies,[27,37] which reported that the fibroblasts resembled many behaviors of MSCs such as cell morphology, self-renewing capacity, and cell surface protein expression, but they lacked multipotency.

It was noted that the CD271+ cells expressed CD73 at the highest levels of 89.9±6.3 % followed by CD105 at 60.6±9.7 % and CD90 at 48.7±16.7 %. Therefore, our results also demonstrated that the CD 271+ cells were co-expressed with CD105, 73, and 90, whereas they negatively expressed the hematopoietic stem cell markers. The result was similar to some previous studies which found that 49.6±1.7 % of CD271+ ADSCs co-expressed with CD90[17] and 99 % of CD 217+ cells co-expressed with CD90 and CD105[38], but contrasted to other studies, which found that only 10-20 % of CD271+ BMSCs co-expressed with CD90.[14,16] Several surface markers have been investigated as co-expression markers of the CD271+ cells. Some studies found that 82–85 % of the CD271+ cells from adipose tissue co-expressed with CD34.[29,38] Maria, *et al.*[39] reported the usefulness of using CD271 combined with CD45 to isolate fresh bone marrow MSCs. Mabuchi, *et al.*[40] suggested that a combination of markers using CD271, CD90, and CD106 for the isolation achieved the most potent and genetically stable MSC.

For differentiation of the cells, the results of our study demonstrated that the ADSCs could differentiate into three lineages including adipogenesis, chondrogenesis, and osteogenesis. Several previous studies investigated the correlations between the CD markers of the cells and their ability to differentiate. Some studies correspondingly demonstrated that MSCs, which are positive to the CD271, 73, and 105, have the potency of chondrogenic differentiation,[41-44] whereas those that are positive to CD90, have more potency for osteogenic differentiation in both *in vitro* and *in vivo*.[45-47] Arufe, *et al.*[41] investigated the differentiation of CD73+ and CD271+ synovial membrane cells and found that the CD271+ cells had higher potency of chondrogenic differentiation compared with CD73+ cells. Ruth, *et al.*[31] found that about 10.6 % of cultured dental pulp cells were positive for CD271 and they had promising odontogenic and chondrogenic potential. The CD105+ cells showed significantly greater chondrogenic potential *in vitro* even when cultured on tissue culture plastic, gel-embedded sheets[42] and biodegradable scaffolds.[43] Kavan, *et al.*[25] reported that CD90+ADSCs underwent improved osteogenic differentiation over CD90-, CD105+, and unsorted cells. *In vitro*, the authors found that co-selection of CD105low+/CD90high+ cells had more osteogenic phenotype compared with CD105low+/CD90low+ cells. Our results showed that although the amounts of the

CD 271+/90+ADSCs were remarkably less than those of the CD271+/73+ and 271+/105+cells, they could differentiate into osteoblastic lineage after culturing in the osteoblastic inductive conditions. Moreover, the levels of osteogenic differentiation markers of the CD271+cells were not statistically different to those of the PA cells. Therefore, it is presumed that the osteogenic differentiation capacity of the CD 271+ADSCs was similar to that of the PA ADSCs.

Various kinds of progenitor cells are present in the perivascular niche of adipose tissue, including tissue-resident mesoderm-derived cells, circulating bone marrow-derived cells, and neural crest (NC)-derived cells.[48] However, the developmental origin of ADSCs still remains unclear. Several literature reports hypothesize that some subsets of bone marrow, dental pulp, and adipose mesenchymal stem cells originate from the neural crest.[49-53] It is found that the neural crest-derived stem cells colonize earlier, but are largely replaced by non-NC derivatives. Therefore, the contribution of NC cells to either BM-MSCs or adipogenic progenitors sharply declines with age and a very small proportion of the NC-derived cells exist in adults.[54-56] Wrage *et al.*[57] reported that approximately 2 % of ADSCs are NC-derived and they do not contribute to neural differentiation under culture conditions. Cuevas-Diaz Duran, *et al.* and Quirici, *et al.,* reported that the amounts of CD271+ cells isolated from fresh human adipose tissue were approximately 2.89 % and 4.4 % respectively.[29,38] Correspondingly, Yoshihiro, *et al.*[58] demonstrated that a minor subpopulation of ADSCs was derived from NC cells and they exhibited an adipocyte-restricted differentiation potential, whereas chondrogenic potential was markedly attenuated. Another theory believes that the perivascular zone is the *in vivo* niche of mesenchymal stem cells which arise from a fibroblastic or pericytic origin. The cells are recognized as pericytes or perivascular cells which reside in the innermost layer of stromal cells contacting vessel endothelium.[27,58-60] CD146 is considered to be an early surface marker of MSCs derived from perivascular cells.[61] CD146+ perivascular cells can express general MSC surface antigens of CD73, CD90, and CD105, and they commonly negatively express CD31, CD34, and CD45.[25,62,63] On the other hand, CD146 is also highly expressed in MSCs, but not in dermal fibroblasts.[63] Feng-Juan, *et al.*[27] suggest that CD146 is another appropriate stem cell marker for universal detection of MSC populations from various tissues. The authors suggested that CD271+/CD146- cells are bone-lining cells, whereas, CD271+/146+ cells have perivascular localization. On the contrary, Yoshihiro, *et al.*[58] identified p75NTR-positive NC-derived cells along the vessels in the trunk fat tissue and found that almost none of them were positive to the pericyte markers. Therefore, the amounts of CD146 should be investigated as the co-expressed marker of CD271+ADSCs in future experiments.

Conclusion

The buccal fat pad is a suitable intra-oral source of mesenchymal stem cells. The CD271 surface marker seems not to be suitable to be used as the single marker for the sorting technique of ADSCs from buccal fat tissue.

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References

1. Chen W, Liu X, Chen Q, Bao C, Zhao L, Zhu Z, *et al.* Angiogenic and osteogenic regeneration in rats via calcium phosphate scaffold and endothelial cell coculture with hBMSCs, hUCMSCs, hiP-SC-MSCs and hESC-MSCs. ***J Tissue Eng Regen Med*** 2017. doi:10.1002/term.2395.[Epub ahead of print]

2. Wongsupa N, Nuntanaranont T, Kamolmattayakul S, Thuaksuban N. Biological characteristic effects of human dental pulp stem cells on poly-epsilon-caprolactone-biphasic calcium phosphate fabricated scaffolds using modified melt stretching and multilayer deposition. ***J Mater Sci Mater Med*** 2017;28:25.

3. Lendeckel S, Jodicke A, Christophis P, Heidinger K, Wolff J, Fraser JK, *et al.* Autologous stem cells (adipose) and fibrin glue used to treat widespread traumatic calvarial defects: case report. *J*

Craniomaxillofac Surg 2004;32:370-3.

4. Diomede F, Zini N, Gatta V, Fulle S, Merciaro I, D'Aurora M, *et al.* Human periodontal ligament stem cells cultured onto cortico-cancellous scaffold drive bone regenerative process. ***Eur Cell Mater*** 2016;32:181-201.

5. Wongsupa N, Nuntanaranont T, Kamolmattayakul S, Thuaksuban N. Assessment of bone regeneration of a tissue-engineered bone complex using human dental pulp stem cells/poly(epsilon-caprolactone)-biphasic calcium phosphate scaffold constructs in rabbit calvarial defects. ***J Mater Sci Mater Med*** 2017;28:77.

6. Kern S, Eichler H, Stoeve J, Kluter H, Bieback K. Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. ***Stem Cells*** 2006;24:1294-301.

7. Farre-Guasch E, Marti-Page C, Hernandez-Alfaro F, Klein-Nulend J, Casals N. Buccal fat pad, an oral access source of human adipose stem cells with potential for osteochondral tissue engineering: an *in vitro* study. ***Tissue Eng Part C Methods*** 2010;16:1083-94.

8. Zuk PA, Zhu M, Ashjian P, De Ugarte DA, Huang JI, Mizuno H, *et al.* Human adipose tissue is a source of multipotent stem cells. ***Mol Biol Cell*** 2002;13:4279-95.

9. Karantalis V, Hare JM. Use of mesenchymal stem cells for therapy of cardiac disease. ***Circ Res*** 2015;116:1413-30.

10. Suzuki E, Fujita D, Takahashi M, Oba S, Nishimatsu H. Adipose tissue-derived stem cells as a therapeutic tool for cardiovascular disease. ***World J Cardiol*** 2015;7:454-65.

11. Bruder SP, Jaiswal N, Haynesworth SE. Growth kinetics, self-renewal, and the osteogenic potential of purified human mesenchymal stem cells during extensive subcultivation and following cryopreservation. ***J Cell Biochem*** 1997;64:278-94.

12. Caplan AI. Adult mesenchymal stem cells for tissue engineering versus regenerative medicine. ***J Cell Physiol*** 2007;213:341-7.

13. Miltenyi S, Muller W, Weichel W, Radbruch A. High gradient magnetic cell separation with MACS. ***Cytometry*** 1990;11:231-8.

14. Kuci S, Kuci Z, Kreyenberg H, Deak E, Putsch K, Huenecke S, *et al.* CD271 antigen defines a subset of multipotent stromal cells with immunosuppressive and lymphohematopoietic engraftment-promoting properties. ***Haematologica*** 2010;95:651-9.

15. Poloni A, Maurizi G, Rosini V, Mondini E, Mancini S, Discepoli G, *et al.* Selection of CD271(+) cells and human AB serum allows a large expansion of mesenchymal stromal cells from human bone marrow. ***Cytotherapy*** 2009;11:153-62.

16. Quirici N, Soligo D, Bossolasco P, Servida F, Lumini C, Deliliers GL. Isolation of bone marrow mesenchymal stem cells by anti-nerve growth factor receptor antibodies. ***Exp Hematol*** 2002;30:783-91.

17. Yamamoto N, Akamatsu H, Hasegawa S, Yamada T, Nakata S, Ohkuma M, *et al.* Isolation of multipotent stem cells from mouse adipose tissue. ***J Dermatol Sci*** 2007;48:43-52.

18. Sacchetti B, Funari A, Michienzi S, Di Cesare S, Piersanti S, Saggio I, *et al.* Self-Renewing Osteoprogenitors in Bone Marrow Sinusoids Can Organize a Hematopoietic Microenvironment. ***Cell*** 2007;131:324-36.

19. Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, *et al.* Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement.***Cytotherapy*** 2006;8:315-7.

20. Arpornmaeklong P, Sutthitrairong C, Jantaramanant P, Pripatnanont P. Allogenic human serum, a clinical grade serum supplement for promoting human periodontal ligament stem cell expansion. ***J Tissue Eng Regen Med*** 2016:142-52.

21. Arpornmaeklong P, Brown SE, Wang Z, Krebsbach PH. Phenotypic characterization, osteoblastic differentiation, and bone regeneration capacity of human embryonic stem cell-derived mesenchymal stem cells. ***Stem Cells Dev*** 2009;18:955-68.

22. Thuaksuban N, Luntheng T, Monmaturapoj N. Physical characteristics and biocompatibility of the polycaprolactone-biphasic calcium phosphate scaffolds fabricated using the modified melt stretching and multilayer deposition. ***J Biomater Appl*** 2016;30:1460-72.

23. Zuk PA, Zhu M, Mizuno H, Huang J, Futrell JW, Katz AJ, *et al.* Multilineage cells from human adipose tissue: implications for cell-based therapies. ***Tissue Eng*** 2001;7:211-28.

24. Bunnell BA, Flaat M, Gagliardi C, Patel B, Ripoll C. Adipose-derived stem cells: isolation, expansion and differentiation. ***Methods*** 2008;45:115-20.

25. Johal KS, Lees VC, Reid AJ. Adipose-derived stem cells: selecting for translational success. ***Regen Med*** 2015;10:79-96.

26. Zimmerlin L, Donnenberg VS, Pfeifer ME, Meyer EM, Peault B, Rubin JP, *et al.* Stromal vascular progenitors in adult human adipose tissue. ***Cytometry A*** 2010;77:22-30.

27. Lv FJ, Tuan RS, Cheung KM, Leung VY. Concise review: the surface markers and identity of human mesenchymal stem cells. ***Stem Cells*** 2014;32:1408-19.

28. Jones E, McGonagle D. Human bone marrow mesenchymal stem cells *in vivo*. ***Rheumatology*** 2008;47:126-31.

29. Quirici N, Scavullo C, de Girolamo L, Lopa S, Arrigoni E, Deliliers GL, *et al.* Anti-L-NGFR and -CD34 monoclonal antibodies identify multipotent mesenchymal stem cells in human adipose tissue. ***Stem Cells Dev*** 2010;19:915-25.

30. Alvarez-Viejo M, Menendez-Menendez Y, Otero-Hernandez J. CD271 as a marker to identify mesenchymal stem cells from diverse sources before culture. ***World J Stem Cells*** 2015;7:470-6.

31. Alvarez R, Lee HL, Hong C, Wang CY. Single CD271 marker

isolates mesenchymal stem cells from human dental pulp. ***Int J Oral Sci*** 2015;7:205-12.

32. Tomlinson MJ, Jones EA, Giannoudis PV, Yang XB, Kirkham J. CD271 Negative Human Dental Pulp Cells Yield Significantly More Adherent Colony Forming Cells than the Positive Phenotype. ***Int J Stem Cell Res Ther*** 2016;3:025.

33. Jarocha D, Lukasiewicz E, Majka M. Adventage of mesenchymal stem cells (MSC) expansion directly from purified bone marrow CD105+ and CD271+ cells. ***Folia Histochem Cytobiol*** 2008;46:307-14.

34. Mitchell JB, McIntosh K, Zvonic S, Garrett S, Floyd ZE, Kloster A, *et al.* Immunophenotype of human adipose-derived cells: temporal changes in stromal-associated and stem cell-associated markers. ***Stem cells*** 2006;24:376-85.

35. Bourin P, Bunnell BA, Casteilla L, Dominici M, Katz AJ, March KL, *et al.* Stromal cells from the adipose tissue-derived stromal vascular fraction and culture expanded adipose tissue-derived stromal/stem cells: a joint statement of the International Federation for Adipose Therapeutics and Science (IFATS) and the International Society for Cellular Therapy (ISCT). ***Cytotherapy*** 2013;15:641-8.

36. Dominici M, Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. ***Cytotherapy*** 2006;8:315-7.

37. Alt E, Yan Y, Gehmert S, Song YH, Altman A, Gehmert S, *et al.* Fibroblasts share mesenchymal phenotypes with stem cells, but lack their differentiation and colony-forming potential. ***Biol Cell*** 2011;103:197-208.

38. Cuevas-Diaz Duran R, González-Garza MT, Cardenas-Lopez A, Chavez-Castilla L, Cruz-Vega DE, Moreno-Cuevas JE. Age-Related Yield of Adipose-Derived Stem Cells Bearing the Low-Affinity Nerve Growth Factor Receptor. ***Stem Cells Int*** 2013;2013:372164.

39. Alvarez-Viejo M, Menendez-Menendez Y, Blanco-Gelaz MA, Ferrero-Gutierrez A, Fernandez-Rodriguez MA, Gala J, *et al.* Quantifying mesenchymal stem cells in the mononuclear cell fraction of bone marrow samples obtained for cell therapy. ***Transplant Proc*** 2013;45:434-9.

40. Mabuchi Y, Morikawa S, Harada S, Niibe K, Suzuki S, Renault-Mihara F, *et al.* LNGFR(+)THY-1(+)VCAM-1(hi+) cells reveal functionally distinct subpopulations in mesenchymal stem cells. ***Stem Cell Reports*** 2013;1:152-65.

41. Arufe MC, De la Fuente A, Fuentes I, de Toro FJ, Blanco FJ. Chondrogenic potential of subpopulations of cells expressing mesenchymal stem cell markers derived from human synovial membranes. ***J Cell Biochem*** 2010;111:834-45.

42. Ishimura D, Yamamoto N, Tajima K, Ohno A, Yamamoto Y, Washimi O, *et al.* Differentiation of adipose-derived stromal vascular fraction culture cells into chondrocytes using the method of cell sorting with a mesenchymal stem cell marker. ***Tohoku J Exp Med*** 2008;216:149-56.

43. Jiang T, Liu W, Lv X, Sun H, Zhang L, Liu Y, *et al.* Potent *in vitro* chondrogenesis of CD105 enriched human adipose-derived stem cells. ***Biomaterials*** 2010;31:3564-71.

44. Rada T, Reis RL, Gomes ME. Distinct stem cells subpopulations isolated from human adipose tissue exhibit different chondrogenic and osteogenic differentiation potential. ***Stem Cell Rev*** 2011;7:64-76.

45. Yamamoto M, Nakata H, Hao J, Chou J, Kasugai S, Kuroda S. Osteogenic Potential of Mouse Adipose-Derived Stem Cells Sorted for CD90 and CD105 *In Vitro*. ***Stem Cells Int*** 2014;2014:1-17.

46. Chung MT, Liu C, Hyun JS, Lo DD, Montoro DT, Hasegawa M, *et al.* CD90 (Thy-1)-Positive Selection Enhances Osteogenic Capacity of Human Adipose-Derived Stromal Cells. ***Tissue Eng Part A*** 2013;19:989-97.

47. Hosoya A, Hiraga T, Ninomiya T, Yukita A, Yoshiba K, Yoshiba N, *et al.* Thy-1-positive cells in the subodontoblastic layer possess high potential to differentiate into hard tissue-forming cells. ***Histochem Cell Biol*** 2012;137:733-42.

48. Majka SM, Fox KE, Psilas JC, Helm KM, Childs CR, Acosta AS, *et al.* De novo generation of white adipocytes from the myeloid lineage via mesenchymal intermediates is age, adipose depot, and gender specific. ***Proc Natl Acad Sci U S A*** 2010;107:14781-6.

49. Billon N, Iannarelli P, Monteiro MC, Glavieux-Pardanaud C, Richardson WD, Kessaris N, *et al.* The generation of adipocytes by the neural crest. ***Development*** 2007;134:2283-92.

50. Mao JJ, Prockop DJ. Stem cells in the face: tooth regeneration and beyond. ***Cell stem cell*** 2012;11:291-301.

51. d'Aquino R, De Rosa A, Laino G, Caruso F, Guida L, Rullo R, *et al.* Human dental pulp stem cells: from biology to clinical applications. ***J Exp Zool B Mol Dev Evol*** 2009;312b:408-15.

52. Navabazam AR, Sadeghian Nodoshan F, Sheikhha MH, Miresmaeili SM, Soleimani M, Fesahat F. Characterization of mesenchymal stem cells from human dental pulp, preapical follicle and periodontal ligament. ***Iran J Reprod Med*** 2013;11:235-42.

53. Stevens A, Zuliani T, Olejnik C, LeRoy H, Obriot H, Kerr-Conte J, *et al.* Human dental pulp stem cells differentiate into neural crest-derived melanocytes and have label-retaining and sphere-forming abilities. ***Stem Cells Dev*** 2008;17:1175-84.

54. Takashima Y, Era T, Nakao K, Kondo S, Kasuga M, Smith AG, *et al.* Neuroepithelial cells supply an initial transient wave of MSC differentiation. ***Cell*** 2007;129:1377-88.

55. Morikawa S, Mabuchi Y, Niibe K, Suzuki S, Nagoshi N, Sunabori T, *et al.* Development of mesenchymal stem cells partially originate from the neural crest. ***Biochem Biophys Res Commun***

2009;379:1114-9.
56. Lemos DR, Paylor B, Chang C, Sampaio A, Underhill TM, Rossi FM. Functionally convergent white adipogenic progenitors of different lineages participate in a diffused system supporting tissue regeneration. ***Stem cells*** 2012;30:1152-62.
57. Wrage PC, Tran T, To K, Keefer EW, Ruhn KA, Hong J, *et al.* The Neuro-Glial Properties of Adipose-Derived Adult Stromal (ADAS) Cells Are Not Regulated by Notch 1 and Are Not Derived from Neural Crest Lineage. ***PLoS One*** 2008;3:e1453.
58. Sowa Y, Imura T, Numajiri T, Takeda K, Mabuchi Y, Matsuzaki Y, *et al.* Adipose Stromal Cells Contain Phenotypically Distinct Adipogenic Progenitors Derived from Neural Crest. ***PLoS One*** 2014;8:e84206.
59. Feng J, Mantesso A, De Bari C, Nishiyama A, Sharpe PT. Dual origin of mesenchymal stem cells contributing to organ growth and repair. ***Proc Natl Acad Sci U S A*** 2011;108:6503-8.
60. Slukvin II, Vodyanik M. Endothelial origin of mesenchymal stem cells. ***Cell cycle*** 2011;10:1370-3.
61. Kawashima N. Characterisation of dental pulp stem cells: a new horizon for tissue regeneration? ***Arch Oral Biol*** 2012;57:1439-58.
62. Vaculik C, Schuster C, Bauer W, Iram N, Pfisterer K, Kramer G, *et al.* Human dermis harbors distinct mesenchymal stromal cell subsets. ***J Invest Dermatol*** 2012;132:563-74.
63. Crisan M, Yap S, Casteilla L, Chen CW, Corselli M, Park TS, *et al.* A perivascular origin for mesenchymal stem cells in multiple human organs. ***Cell stem cell*** 2008;3:301-13.

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