Anti-cariogenic Effect of Experimental Resin Cement Containing Ursolic Acid Using Dental Microcosm Biofilm

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Anti-cariogenic Effect of Experimental Resin Cement Containing Ursolic Acid Using Dental Microcosm Biofilm

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June 2020

감사의 글

본 논문을 쓸 수 있게 기회를 주시고 수개월의 기간 동안 처음부터 끝까지 열정과 관심으로 지도해주신 박정원 교수님께 진심으로 감사드립니다. 또한 논문 심사를 맡아 꼼꼼하고 세심하게 가르쳐 주시고 조언해주신 신수정 교수님, 김백일 교수님께도 감사의 말씀을 올립니다.

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또한 이번 연구에 도움을 주신 박선규 선생님, 저의 동기가 없어 고생 많이 한 의국원들, 많은 조언과 도움을 아끼지 않은 의국 선배님들에게도 감사의 말을 전합니다.

마지막으로 저를 위해 묵묵히 도와주신 부모님을 포함한 가족과 함께 이 기쁨을 나누고 싶습니다.

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조 종 현

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Abstract

Anti-cariogenic Effect of Experimental Resin Cement Containing Ursolic Acid Using Dental Microcosm Biofilm

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(Directed by Professor Jeong-Won Park, D.D.S., M.S.D., Ph.D.)

Secondary caries was reported as the main cause of replacement of composite resin restorations. To solve this problem, there have been numerous attempts to add anti-cariogenic materials into composite resin. Among those materials, ursolic acid (UA) was reported to inhibit the growth of cariogenic microorganisms and biofilm formation, which suggests that UA have considerable antibacterial agents for dental caries prevention.

Furthermore, anti-carious effect of UA around the composite resin restoration was reported consequently.

In previous study, experimental resin cement containing UA was also reported to exert anti-cariogenic activity against *S.mutans*. However, there has been no study to evaluate the anti-cariogenic activity of resin cement against dental microcosm biofilms so far. Thus, this study was designed to evaluate anti-cariogenic activity of resin cement containing ursolic acid (UA) against caries-related microcosm biofilms and to determine the optimal concentration of UA.

Experimental resin cement was prepared according to UA concentration (0, 0.1, 0.5, 1.0 and 2.0 wt%). Fifty extracted human molars were prepared with a 2 mm x 4 mm x 2 mm (M-D width x B-L width x depth) cavity on the occlusal surface. For each sample, an indirect resin inlay was made of Tescera^{TM} system (Body A1 shade, Bisco, Schaumberg, IL, USA) following the manufacturer's instructions. Then, Indirect resin inlays were cemented with experimental resin cement for each sample. Acid-resistant nail varnish (Wakemake, Seoul, Korea) was applied, except for the area 2 mm around the restoration.

Dental microcosm biofilms were initiated from human saliva on extracted human molars to reflect conditions that are relevant to dental caries. Before biofilm growing, bacterial composition of human saliva was analyzed by 16s RNA microbiome profiling. Bacterial composition was shown normal range of oral microflora. There was no evidence of contamination in human saliva inoculum. Biofilms were then grown for 10 days in basal

medium mucin (BMM) artificial saliva medium to induce artificial caries. To evaluate the caries progression, Quantitative Light-induced Fluorescence (QLF) was used to compare the before and after caries induction. One-way ANOVA followed by the Tukey post-hoc analysis was used to statistically analyze the data ($p < 0.05$).

ΔF (-%) represents the relative mean loss of fluorescence of lesion comparing to sound enamel, the more negative value means the greater mineral loss and caries progression. ΔQ (-%·Px) represents total fluorescence loss volume of the lesion area. It was calculated as a multiplication of the lesion area (pixel) and the mean change in fluorescence (-%).

On ΔF (-%) and ΔQ (-%·Px) values as QLF parameters, there was no significant ΔF changes after caries induction. Otherwise, there was a tendency of ΔQ (-%·Px) changes after caries induction being lower in groups of resin cement containing higher concentration of UA. The difference between before and after caries induction of ΔQ in control group was -3660.6 but the value of 2% group was only -404.8. It means that artificial caries was less induced in the area around resin cement containing UA of more than or equal to 1.0% significantly ($p<0.05$). There was no difference between the groups containing UA of more than or equal to 1.0%. In the previous study that evaluate anti-cariogenic activity against *S.mutans*, resin cement containing UA of more than or equal to 0.5% showed statistically significant anti-cariogenic activity.

Within the limitations of this study, resin cement containing at least 1.0% of UA showed an anti-cariogenic effect.

Keywords : Anti-cariogenic resin cement, Ursolic acid, dental microcosm biofilm

Anti-cariogenic Effect of Experimental Resin Cement Containing Ursolic Acid Using Oral Microflora

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I. Introduction

Resin composite materials exhibit good esthetic properties and strength, which has gained wide popularity in dental practice in the past few decades (Haj-Ali, et al. 2005). However, many studies indicated numerous failures occurred (Heintze and Rousson 2012; Manhart, et al. 2004) and among those failures, secondary caries was reported as the main cause of replacement of composite resin restorations (Burke, et al. 2001; Haj-Ali, et al. 2005; Moraschini, et al. 2015).

To inhibit secondary caries and increase survival rate of resin composite restorations, there have been numerous attempts including addition of silver nanoparticles, quaternary ammonium polyethyleneimine nanoparticles, chlorhexidine diacetate, mesoporous SiO2, cellulose nanocrystal/zinc oxide (CNC/ZnO), chitosan (Ali, et al. 2020; Beyth, et al. 2006; Fan, et al. 2011; Imazato 2009; Kozai, et al. 1987; Leung, et al. 2005; Liu 1995; Liu 2005; Wang, et al. 2019; Zhang, et al. 2018). However, these experimental antibacterial composite resins exhibited several limitations, such as decreased anti-bacterial effect over time, compromised physical property and toxicity to normal cell (Ali, et al. 2020; Fan, et al. 2011; Hiraishi, et al. 2008). Not yet, none of them have been clinically used.

Ursolic acid (UA) is a natural triterpenoid compound with antibacterial, anti-inflammatory and anti-tumor effects (Liu 1995; Liu 2005). Because of its hydrophobic property, it can be blended with the resin matrix and is not easily eluted in the saliva, a feature that is anticipated to provide antibacterial durability (Kim, et al. 2013). In previous studies, composite resin containing UA inhibited the growth of cariogenic microorganisms and biofilm formation, which suggests that UA have considerable antibacterial agents for dental caries prevention (Zhou, et al. 2013; Zou, et al. 2014). Furthermore, anti-carious effect of UA around the composite resin restoration was reported consequently (KIm, et al. 2011; Kim, et al. 2013).

According to previous study, the UA incorporated in monomer groups were more anti-bacterial method than the UA-coated filler groups (Kim 2013). So, there was an attempt to incorporate UA into resin cement rather than composite resin. Resin cement has lower

flexural strength than conventional composite resin because it has lower filler contents (Baroudi and Rodrigues 2015). Thus, it can contain more UA and can be anticipated to have an enhanced anti-bacterial effect. Previous study reported that resin cement containing UA showed an anti-carious effect and satisfied ISO criteria for flexural strength, film thickness and in vitro cytotoxicity (Yoo, et al. 2019).

Because *Streptococcus mutans* have been identified as the major pathogens of dental caries, in previous study artificial caries were induced from biofilm through *Streptococcus mutans* (Yoo, et al. 2019). But, several studies have revealed that the level of *Streptococcus mutans* was not necessarily high in caries-associated biofilms, especially the microflora associated with non-cavitated stages of lesion formation (Sansone, et al. 1993).

Non-mutans acidogenic and aciduric bacteria, including non-mutans streptococci and *Actinomyces*, were proposed to be more involved with the initiation of caries rather than *S. mutans* (van Ruyven, et al. 2000). Caries lesion formation is not solely associated with *S. mutans* single species. Instead, biofilm properties are determined by interactions between internal bacteria. Artificial biofilms composed of a single or only a few species do not represent the diversity, complexity, and heterogeneity of in vivo plaque. So, it is vital to reproduce the natural microbial ecology as possible to validate the anti-carious activity of ursolic acid.

For these reasons, the dental microcosm biofilm formed from saliva inoculum, which is a resource of natural oral microflora, has been used in previous laboratory studies (Azevedo,

et al. 2011; Sissons 1997; Tang, et al. 2003). Also, in present study, we intended to induce artificial caries through caries-related biofilms that initiated from human saliva to reproduce *in vivo* caries-related biofilms.

Quantitative light-induced fluorescence (QLF) is a new method for early caries detection in dentistry. This new method uses the principle of fluorescence for visual enhancement of caries detection. The method relies on contrast differences between sound and demineralized tooth by fluorescence. The observed natural fluorescence of a tooth is decreased due to increased scattering when a carious lesion is present (de Josselin de Jong, et al. 1995; van der Veen and de Josselin de Jong 2000).

So, through analyzing fluorescence loss of picture of tooth it can produce the fluorescence parameters which allow comparison between the longitudinal tooth states without destruction of tooth (Aljehani, et al. 2006; de Josselin de Jong, et al. 1995). It has been reported as sensitive and effective to detect changes of mineral loss of early caries (Kang, et al. 2017). It has been used for evaluation of longitudinal changes in de/remineralization (Kim and Kim 2018a; Kim and Kim 2018b).

In previous study (Yoo, et al. 2019), the anti-cariogenic activity of experimental resin cement containing UA was evaluated against *S.mutans.* To represent clinical situation, present study was designed to evaluate the anti-cariogenic activity of the same experimental resin cement containing UA against dental microcosm biofilm model. The only difference

between studies was the bacteria used. There has been no study to evaluate the anti-cariogenic activity of resin cement against dental microcosm biofilms so far.

Therefore, the aim of this study was to evaluate the anti-carious activity of resin cement containing UA, and determine the optimal UA concentration for anti-carious resin cement in microcosm biofilm model. The null hypotheses were as follows.

1. There was no difference in the caries progression in all experimental groups.
2. There was no optimal UA concentration for anti-carious resin cement.

II. Materials and methods

1. Preparation of experimental resin cements

Five different concentrations of experimental resin cement containing UA were prepared (0, 0.1, 0.5, 1.0, and 2.0 wt%). The concentrations of UA was selected as with the previous study (Yoo, et al. 2019), in which the significant concentration of UA for anti-cariogenic activity was 0.5%. Control group was 0% UA group because there was no ursolic acid in the experimental resin cement in 0% UA group. The composition of the experimental resin cements was displayed in Table 1.

Followings are the components of experimental resin cement. Bisphenol A-glycidyl methacrylate (Bis-GMA), Triethyleneglycol dimethacrylate (TEG-DMA), Camphoroquinone (CQ) as light initiator, Ethyl-4-dimethylamino benzoate (EDMAB) as tertiary amine activator, monoethyl etherhydroquinone (MEHQ) as inhibitor. Filler consisted of 0.803 μm sized Al_2O_3, BaO, B_2O_3, SiO_2 and 0.655 μm sized YbF_3. Matrix and filler compositions were 50:50. Antibacterial ursolic acid was mixed in matrix depending on weight % concentration (Table 1).

Table 1. The components of experimental resin cement by group (wt%)

	Components	Control	UA 0.1	UA 0.5	UA 1.0	UA 2.0	Subtotal
Matrix	Bis-GMA	24.37	24.33	24.13	23.88	23.38	50
	TEGDMA	24.37	24.33	24.13	23.88	23.38	
	CQ	0.4	0.4	0.4	0.4	0.4	
	EDMAB	0.8	0.8	0.8	0.8	0.8	
	MEHQ	0.05	0.05	0.05	0.05	0.05	
	UA	0	0.1	0.5	1	2	
Filler	Al2O3, BaO, B2O3, SiO2	45	45	45	45	45	50
	YbF3	5	5	5	5	5	

Bis-GMA; Bisphenol A-glycidyl methacrylate, TEG-DMA; Triethyleneglycol dimethacrylate, CQ; Camphoroquinone, EDMAB; Ethyl-4-dimethylamino benzoate, MEHQ; monoethyl ether hydroquinone, UA; Ursolic acid.

2. Preparation of tooth specimens

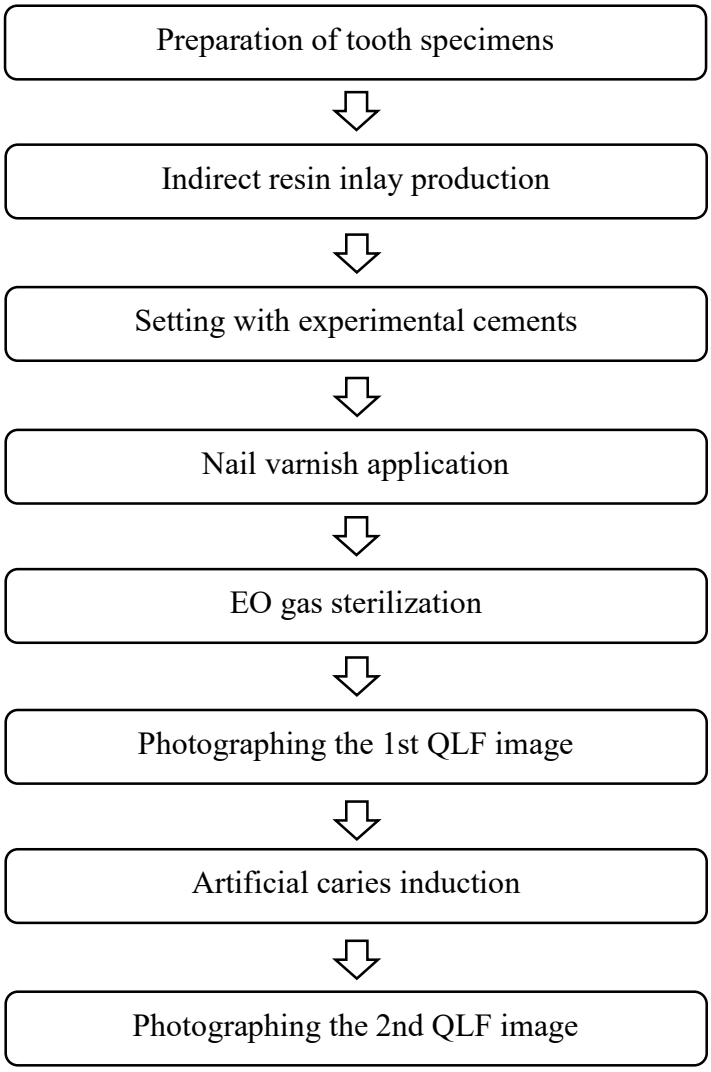

Figure 1. Flow diagram of the evaluation for caries inhibitory effect.

Fifty extracted human molars were collected after obtaining informed consent under a protocol approved by the Gangnam Severance hospital (IRB approval no:3-2020-0047). All tooth specimens were prepared with a 2mm x 4mm x 2mm (M-D width x B-L width x depth) cavity on the occlusal surface. For each sample, an indirect resin inlay was made of the Tescera^{TM} system (Body A1 shade, Bisco, Schaumberg, IL, USA) following the manufacturer's instructions. Then, the resin inlay was set in with the experimental resin cement. Overall flow diagram of the evaluation for caries inhibitory effect was showed in figure 1. Followings are setting procedures.

37% phosphoric acid etching gel was used for prepared cavity for 10 seconds. All-bond universal (Bisco) was applied following the manufacturer's instructions, and then cured for 10 seceonds with Smartlite Focus (950 mW/cm2, Dentsply Sirona, DeTrey, Konstanz, Germany). All-bond universal was applied to the internal surface of the resin inlay and not cured. With the resin cement was applied in the preparation cavity and resin inlay, the inlay was setting in the cavity completely. After tack curing for 2 seconds, excessive resin cement was removed with explorer. Then, all specimens were light cured for 40 seconds. Polishing procedures were done with a silicone rubber point (Jiffy rubber, Ultradent Products Inc, South Jordan, UT, USA) and check the margin of the resin inlay under microscope to remove excessive resin cement totally. Acid resistant nail varnish (Wakemake, Seoul, Korea) was covered on the occlusal surface, except for the restoration and around 2 mm from the resin inlay margin. Sterilization was done with ethylene oxide (EO) gas and QLF

photographs were taken with QLF-D Biluminator™ 2 (Inspektor Research Systems BV, Amsterdam, The Netherlands) before caries induction.

3. Artificial caries induction

To induce artificial caries, biofilms were formed in caries-related microcosm biofilm model as described in detail by Filoche et al.(Filoche, et al. 2007), but modified for this study, in which 24-well cell culture plates and human extracted teeth were used. Approximately 1.5ml of the prepared human saliva was inoculated onto the specimens in each well of the 24-well cell culture plates, and the plates were incubated anaerobically at 37℃ for 4 hours. The saliva was gently aspirated from the base of the wells, and 1.5ml of growth medium that contained BMM and sucrose 0.5% sucrose was added to each well.

The BMM artificial saliva medium contained 2.5 g/l porcine mucin (type III, Sigma Chemicals, MO, USA), 10.0 g/l proteose peptone, 5.0 g/l trypticase peptone, 5.0 g/l yeast extract, 1 mmol/l urea, 1 mmol/l arginine, 2.5 g/l KCl, and 1 mg/l menadione (pH 7.0) (Wong and Sissons 2001). The plates were then incubated under an anaerobic hood within an atmosphere of 80% N_2, 10% CO_2, and 10% H_2 at 37℃ for 10 days and the growth medium was replaced daily.

4. QLF Analysis of caries induction

QLF image were taken before caries induction with QLF-D Biluminator™ 2 (Inspektor Research Systems BV, Amsterdam, The Netherlands). After artificial caries induction, the demineralized specimens were photographed again. The area of analysis was defined as the lesion 1mm around the restorations. Fluorescence losses of white spot lesions were quantified using image analysis software QA2J (version 2.0.0.18, Inspektor Research Systems BV, The Netherlands). This program produced the data as ΔF (-%), lesion size (pixel), and ΔQ (-% · Px). QLF images were analyzed and QLF parameters such as ΔF (-%) and ΔQ (-% · Px) were compared before and after caries induction.

ΔF (-%) represents the relative mean loss of fluorescence of lesion comparing to sound enamel, the more negative value means the greater mineral loss and caries progression. ΔQ (-% · Px) represents total fluorescence loss volume of the lesion area. It was calculated as a multiplication of the lesion area (pixel) and the mean change in fluorescence (-%).

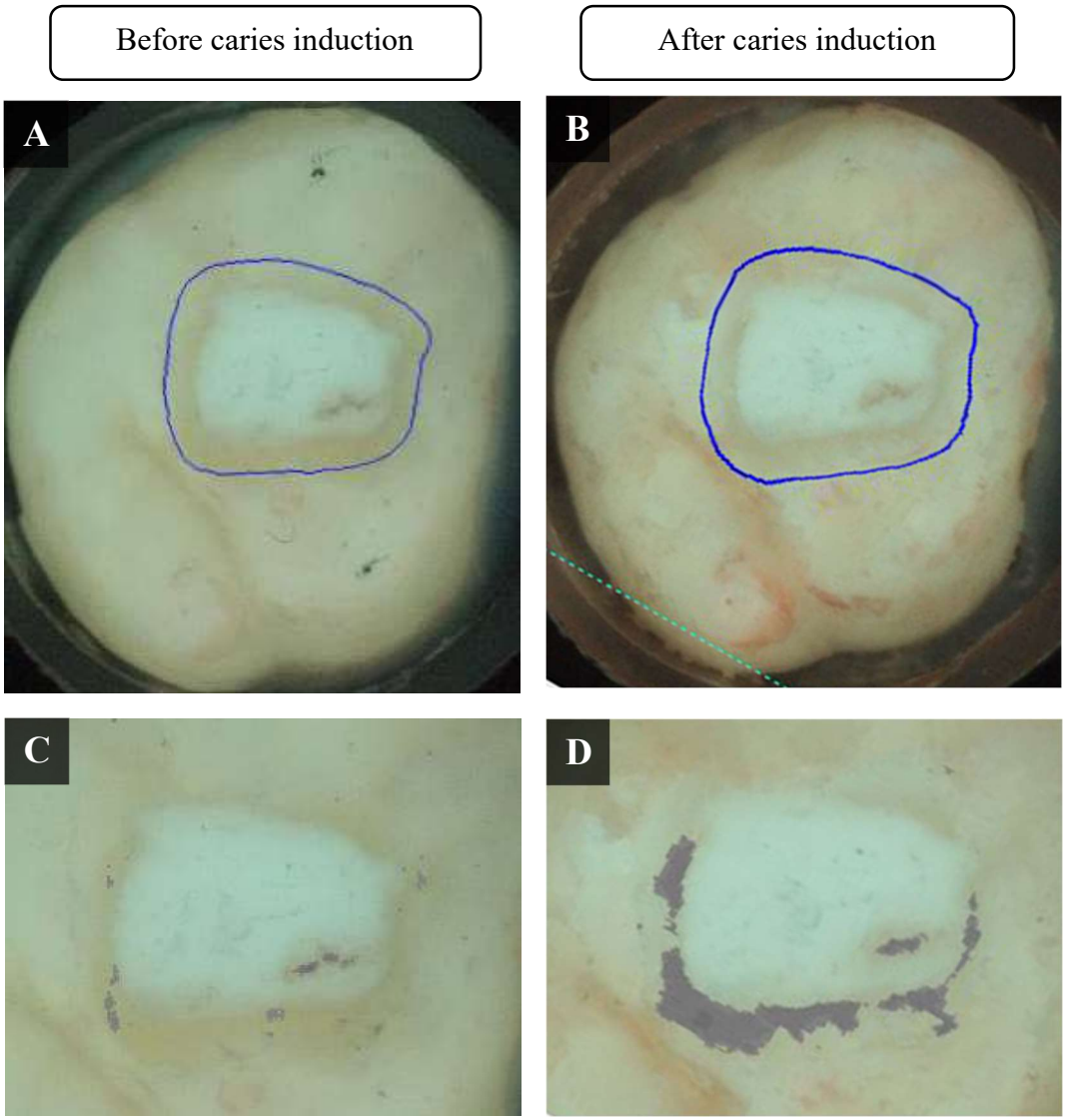

Figure 2. Representative QLF images of before and after caries induction. The areas in the blue lines (A and B) were analyzed. The intensity of dark shadow (C and D) represented fluorescence loss of the lesion whereas the area of dark shadow (C and D) represented the volume of fluorescence loss of the lesion. Through these QLF images, software quantified the relative mean loss of fluorescence (ΔF) and total fluorescence loss volume (ΔQ).

5. Statistical analysis

The difference in ΔF and ΔQ between before and after artificial caries induction indicate loss of fluorescence of lesion during caries induction, which means how much artificial caries is induced. If the UA in experimental resin cement can inhibit the caries progression, the difference in ΔF and ΔQ between before and after caries induction will decrease as UA concentration increase. Thus, we would like to statistically compare the difference in ΔF and ΔQ before and after caries induction according to UA concentration.

One-way ANOVA followed by the Tukey's method for post-hoc analysis was used to statistically analyze the data. Statistical analyses were performed using SAS software version 9.3 (SAS Institute Inc., Cary, NC, USA). An adjusted p value of less than 0.05 was considered to be statistically significant.

6. Bacterial analysis of human saliva

1) DNA Extraction, PCR amplification and Sequencing

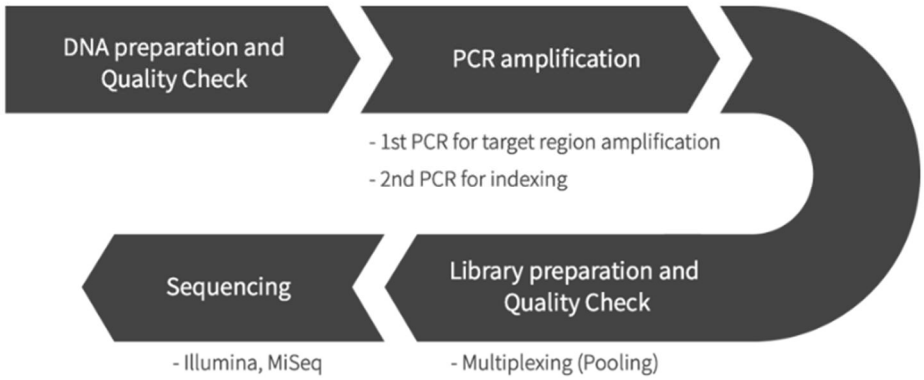

Figure 3. Flow diagram of experimental summary.

Bacterial composition of human saliva inoculum used as inoculum was analyzed. Polymerase Chain Reaction (PCR) amplification method followed by 16s RNA microbiome profiling for taxonomic assignment was performed. Overall flow diagram of bacterial analysis was shown in figure 3.

Total DNA was extracted using the FastDNA® SPIN Kit for Soil (MP Biomedicals, USA), by the manufacturer's instruction. With the extracted DNA PCR amplification was done using fusion primers targeting from V3 to V4 regions of the 16S rRNA gene. To amplify bacteria, fusion primers of 341F (5'-AATGATACGGCGACCACCGAGATCTACAC-XXXXXXXXTCGTCGGCAGCGTC-

AGATGTGTATAAGAGACAG-CCTACGGGNGGCWGCAG-3'; underlining sequence indicates the target region primer) and 805R (5'-CAAGCAGAAGACGGCATACGAGAT-XXXXXXXXGTCTCGTGGGCTCGG-AGATGTGTATAAGAGACAG-GACTACHVGGGTATCTAATCC-3'). The Fusion primers were assembled in the following sequence, which is P5 (P7) graft binding, i5 (i7) index, Nextera consensus, Sequencing adaptor, and Target region sequence.

The amplifications procedures were carried out under the following conditions: initial denaturation at 95 °C for 3min, followed by 25 cycles of denaturation at 95 °C for 30 sec, primer annealing at 55 °C for 30 sec, and extension at 72 °C for 30 sec, with a final elongation at 72 °C for 5 min. with 1% agarose gel electrophoresis the PCR product was established and visualized under a Gel Doc system (BioRad, Hercu les, CA, USA). After purifying the amplified products with the CleanPCR (CleanNA), equal concentrations of purified products were pooled together and eliminated short fragments (non-target products) with CleanPCR (CleanNA). The quality and product size were checked on a Bioanalyzer 2100 (Agilent, Palo Alto, CA, USA) using a DNA 7500 chip. Then, mixed amplicons were pooled and the sequencing was carried out at Chunlab, Inc. (Seoul, Korea), with Illumina MiSeq Sequencing system (Illumina, USA) according to the manufacturer's instructions.

2) Data analysis pipeline

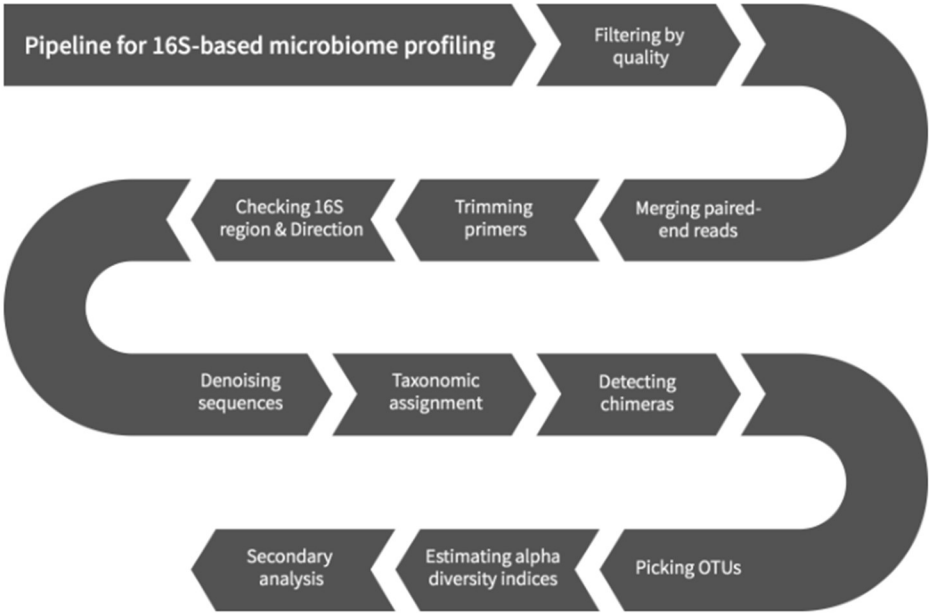

Figure 4. Flow diagram of Data analysis pipeline.

Raw reads were processed with quality check and filtered low quality (<Q25) reads by Trimmomatic ver. 0.32 (Bolger, et al. 2014). Following quality check, paired-end sequence data were merged by fastq_mergepairs command of VSEARCH version 2.13.4 (Rognes, et al. 2016) with default parameters. Then, the primers were trimmed with the alignment alignment algorithm of Myers & Miller (Myers and Miller 1988) at a similarity cut off of 0.8. By nhmmer (Wheeler and Eddy 2013) in HMMER software package ver. 3.2.1 with hmm profiles, non-specific amplicons that do not encode 16s RNA were revealed. Furthermore, other such unique reads were extracted and by utilizing derep_full length command of VSEARCH (Rognes, et al. 2016), redundant reads were grouped with the

unique reads. Before a more precise pairwise alignment was conducted, Taxonomic assignment was done based on the EzBioCloud 16S rRNA database (Yoon, et al. 2017) using usearch_global command of VSEARCH (Rognes, et al. 2016). Subsequently, through UCHIMEUCHIME algorithmalgorithm (Edgar, et al. 2011) and the non-chimeric 16S rRNA database from chimeric 16S rRNA database of EzBioCloud, chimeric reads with <97% similarity by reference-based chimeric detection were filtered. Reads that were not identified to the species level (with <97% similarity) in the database mentioned above were grouped together, and by utilizing cluster_fast command (Rognes, et al. 2016), de-novo clustering was performed in order to produce additional OTUs. Lastly, OTUs with single reads (singletons) were excluded from further analysis. Using in-house programs of Chunlab, Inc (Seoul, South Korea), the secondary analysis including diversity calculation and biomarker discovery was conducted. The alpha diversity indices (ACE (Chao and Lee 1992), Chao (Chao 1987), Jackknife (Burnham and Overton 1979), Shannon (Magurran 2013), NPShannon (Chao 2003), Simpson (Magurran 2013) and Phylogenetic diversity (Faith 1992)), rarefaction curves (Heck, et al. 1975), and rank abundance curves (Whittaker 1965) were estimated. In order to visualize differences between the samples, beta diversity distances were calculated by various algorithms (Jensen-Shannon (Lin 1991), Bray-Curtis (Beals 1984; Chen, et al. 2012), Generalized UniFrac (Chen, et al. 2012), Fast UniFrac (Hamady, et al. 2010)). Taxonomic and functional biomarkers were discerned via statistical comparison algorithms (LDA Effect Size - LEfSe (Segata, et al. 2011) and Kruskal-Wallis H Test (Kruskal 1952)) as well as functional profiles that were predicted by PICRUSt (Ye

2009) and MinPath (Langille, et al. 2013) algorithms. All of the analytics mentioned above were performed in EzBioCloud 16S-based MTP, a ChunLab's bioinformatics cloud platform.

7. Micro-CT analysis of tooth specimen

Micro-CT images were taken after caries induction using SkyScan 1173 (Bruker corporation, Billerica, USA). 2 specimens for each group (0, 0.1, 0.5, 1.0 and 2.0% UA group) were selected for micro-CT analysis. X-ray source was 130kV, 8W, 5um spot size and spatial resolution was 4-5um detail detectability. The density of caries induced area around restoration was analyzed by gray scale compared to sound enamel surface using image software Image J (National Institutes of Health, Bethesda, Maryland, USA). Gray scales of bilateral enamel surface 50 pixels from restoration margin were averaged and compared to the gray scale of sound enamel area. The gray scale of sound enamel surface was analyzed by histogram.

III. Results

1. Caries inhibitory effect

After caries induction, white spot lesions formation was shown around the restoration on white light image and tooth color on QLF image changed according to fluorescence loss (Figure 5). The absolute mean fluorescence loss (ΔF) and the total fluorescence loss volume (ΔQ) increased after caries induction compared with the before caries induction state based on one-way ANOVA followed by the Tukey's method for post-hoc analysis ($p<0.05$) (Figure 6). It means tooth samples were demineralized through caries induction process. The differences between before and after caries induction state appeared more notably in ΔQ than ΔF.

The measured results and P-values are represented in Table 2. The difference between before and after caries induction of ΔF ranged from -1.36 to -0.42 and ΔQ ranged from -3660.6 to -404.77. All values in experimental group changed more negatively and the difference of data were more pronounced in ΔQ than ΔF. The difference of ΔF and ΔQ data in groups of resin cement containing UA between before and after caries induction were lower than the control group (0%) generally. But there was only significant difference on the data of ΔQ in experimental groups containing UA more than 1.0% compared to control.

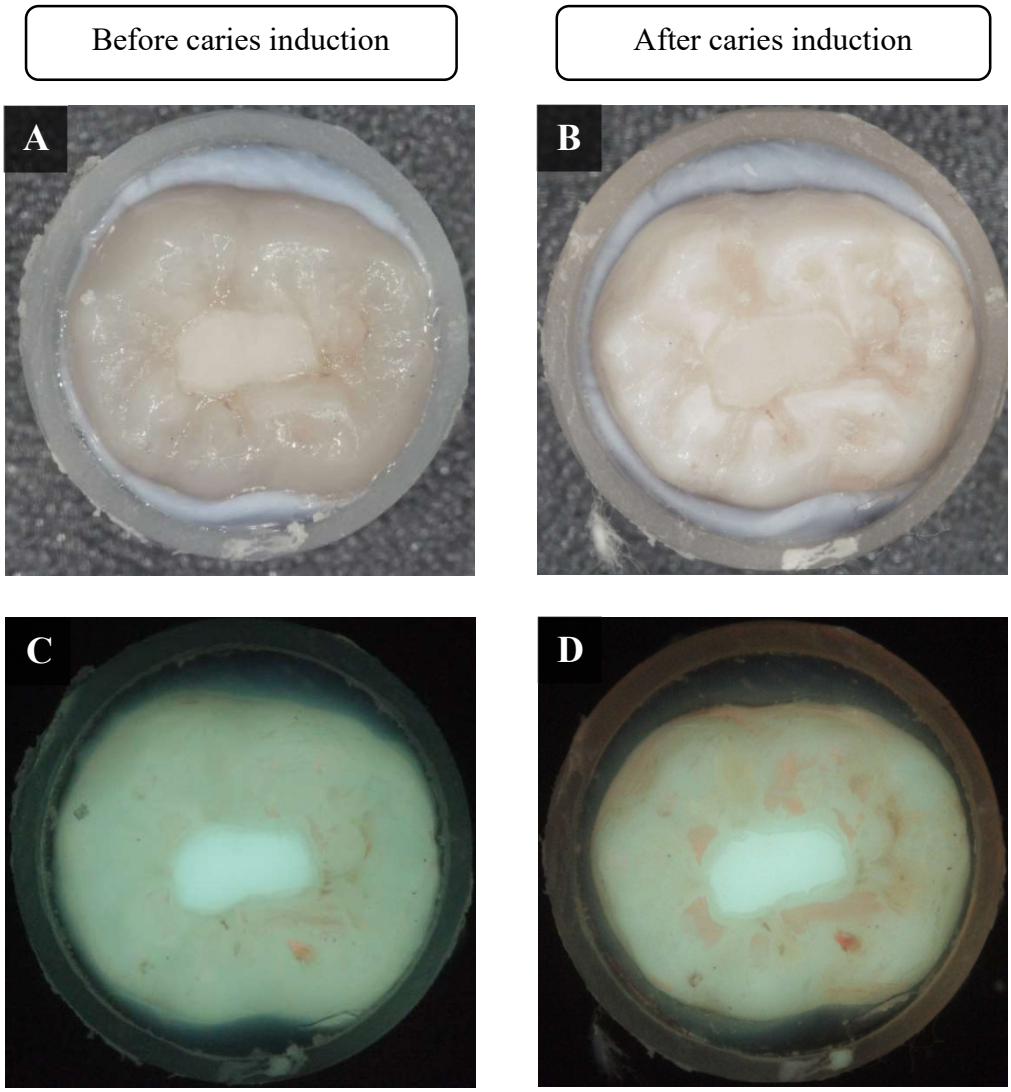

Figure 5. Representative images of before and after caries induction state. White light images (A and B) and QLF images (C and D) are shown. After caries induction, white spot lesion formation was presented around restoration (B) and changes of tooth color around restoration was presented on QLF image comparing to before caries induction state (D)

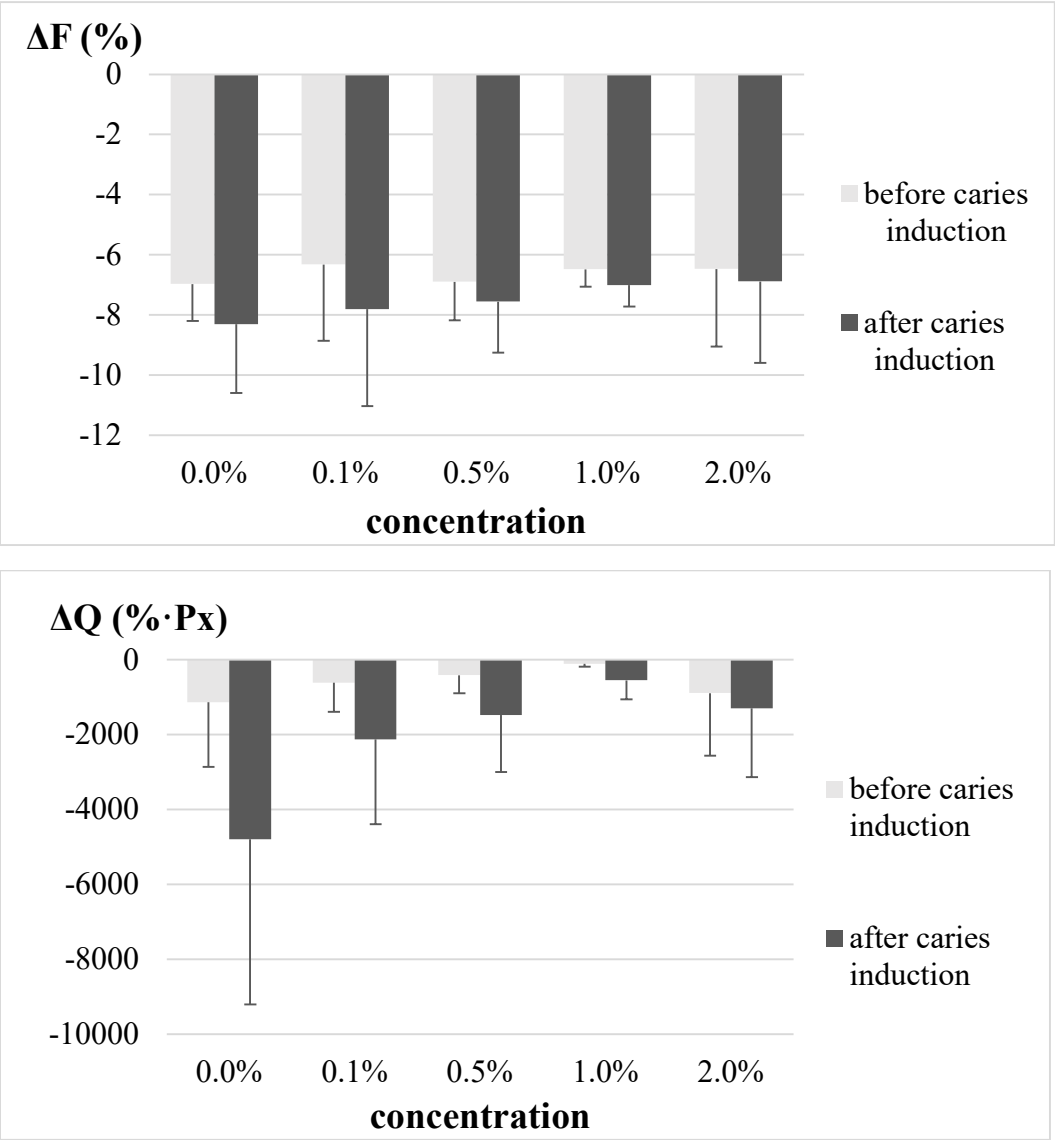

Figure 6. The comparison of ΔF (-%) and ΔQ (-%·Px) between before and after caries induction state. Before caries induction, ΔF and ΔQ values were not significantly different ($p>0.05$). Absolute value of ΔF and ΔQ increased after caries induction comparing to before caries induction, it means that the caries lesions were progressed.

Table 2. the difference between before and after caries induction of ΔF (-%) and ΔQ (-%·Px) by UA concentration (wt%). (n=10)

UA concentration (wt%)	The difference of ΔF (-%)		The difference of ΔQ (-%·Px)	
	Mean±S.D	P-value	Mean±S.D	P-value
0	-1.36±2.06[a]		-3660.6±1509.4[a]	
0.1	-1.48±1.50[a]		-1515.0±1749.5[a]	
0.5	-0.66±1.36[a]	0.538	-1060.4±1213.8[a]	0.027
1.0	-0.53±0.32[a]		-440.5±476.6[b]	
2.0	-0.42±0.63[a]		-404.77±356.4[b]	

ΔF; florescence loss (-%), ΔQ; total fluorescence loss volume of lesion (-%·Px).

a,b: Statistically significant differences between the groups in the same column according to Tukey's post-hoc test.

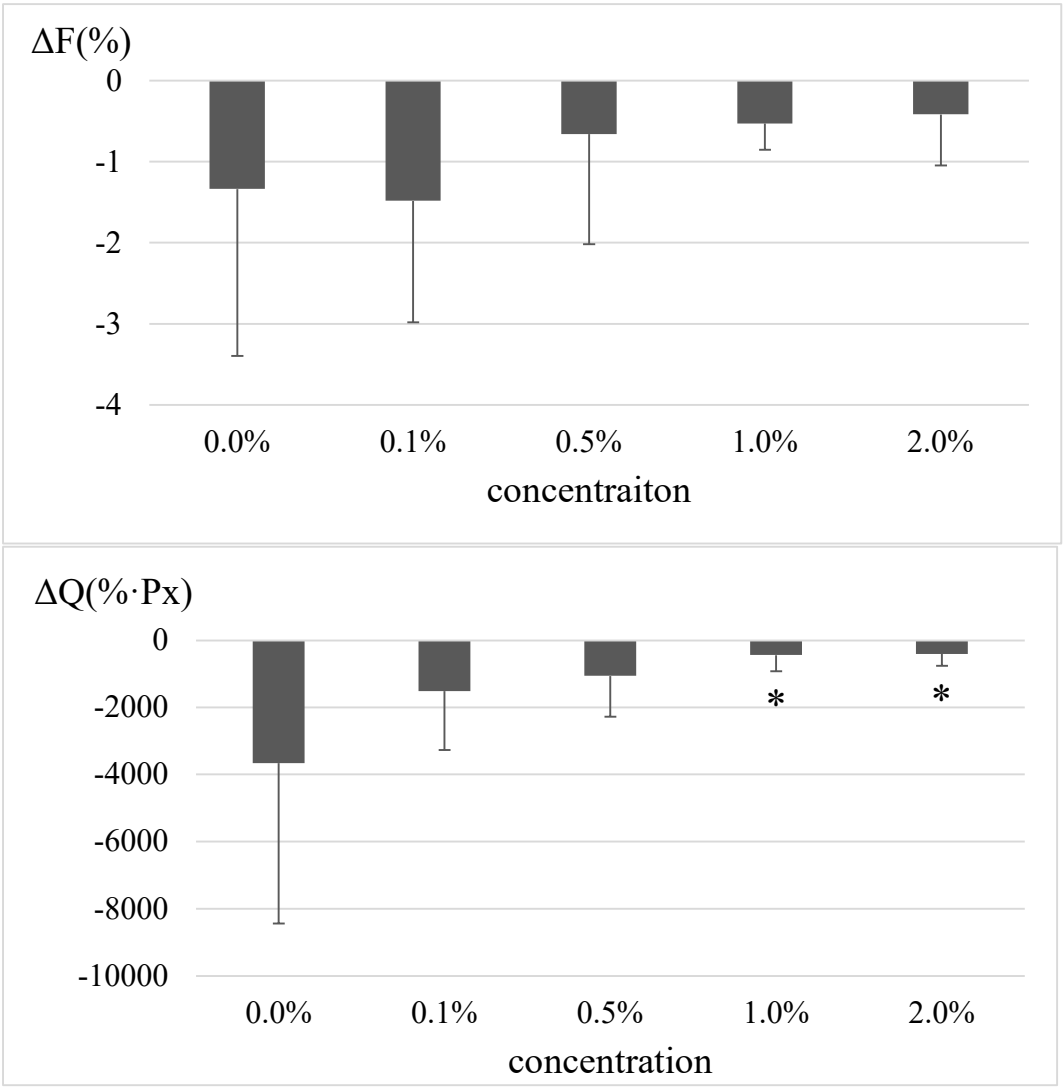

Figure 7. The differences of ΔF (-%) and ΔQ (-%·Px) between before and after caries induction. The differences of ΔF and ΔQ in groups of resin cement containing UA were lower than the control group (0%), but there was only statistically significant difference on difference of ΔQ in experimental groups containing UA at more than or equal to 1.0% compared to with control group. Asterisks (*) represent the statistically significant difference ($p<0.05$).

2. Bacterial analysis of human saliva

After PCR amplification, total reads count was 90,449. Among them, 9,319 reads were removed due to low quality amplicons or chimeric amplicons. Thus, total valid reads count was 81,130. The species analyzed from the human saliva used in present study were shown in Table 3.

Table 3. Taxon composition list of human saliva inoculum. Species under 1% proportion were unclassified in higher taxonomic rank.

#	Taxon name	Count	Proportion(%)
1	Haemophilus parainfluenzae group	23,685	29.1939
2	Streptococcus sanguinis group	13,327	16.4267
3	Neisseria sicca group	7,446	9.1779
4	Neisseria subflava	7,195	8.8685
5	Streptococcus pneumoniae group	5,552	6.8433
6	Rothia aeria	3,607	4.4460
7	Lautropia mirabilis	2,587	3.1887
8	Prevotella melaninogenica	2,575	3.1739
9	Veilonella rogosae	1,592	1.9623
10	Veilonella dispar	1,581	1.9487
11	Fusobacterium periodonticum group	1,432	1.7651
12	Streptococcus sinensis group	1,323	1.6307
13	Streptococcus parasanguinis group	1,150	1.4175
14	Streptococcus peroris group	971	1.1968
15	Etc (<1.0%)	6,095	7.51

3. Micro-CT analysis of tooth specimen

Representative micro-CT image was shown in figure 8 and plot profile was shown in figure 9. As gray value increased, shade on micro-CT image was shown brighter. The difference between averaged gray value of enamel surface 50 pixels from restoration margin and sound enamel surface were shown in figure 10. As UA concentraiton increased, the differnce of gray value was decreased. Averaged Δgray value was -4.88 in 2.0% UA group, whereas -10.54 in control group (0% UA). There was a tendency that the difference of gray value was decreased as UA concentration increased. However, statistical analysis was not performed because of lower sample count (n=2).

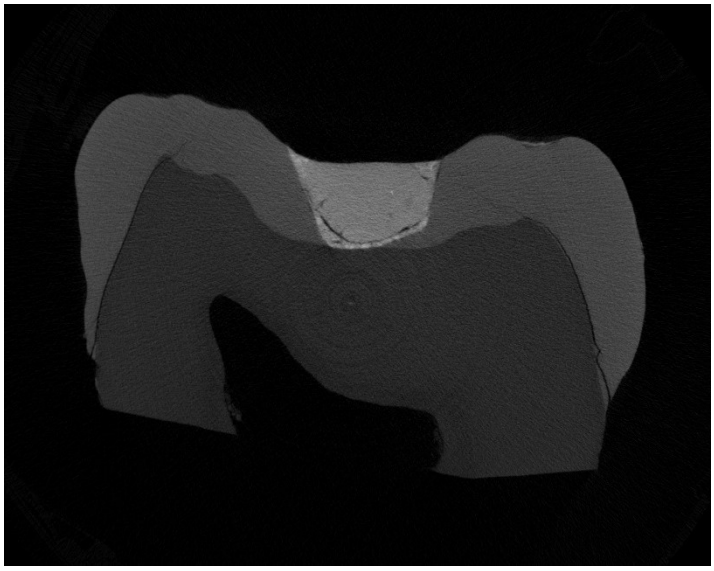

Figure 8. Representative micro-CT image of tooth specimen after caries induction. Bilateral enamel surface 50 pixels from restorations were analyzed by gray scale and compared to the gray scale of sound enamel area.

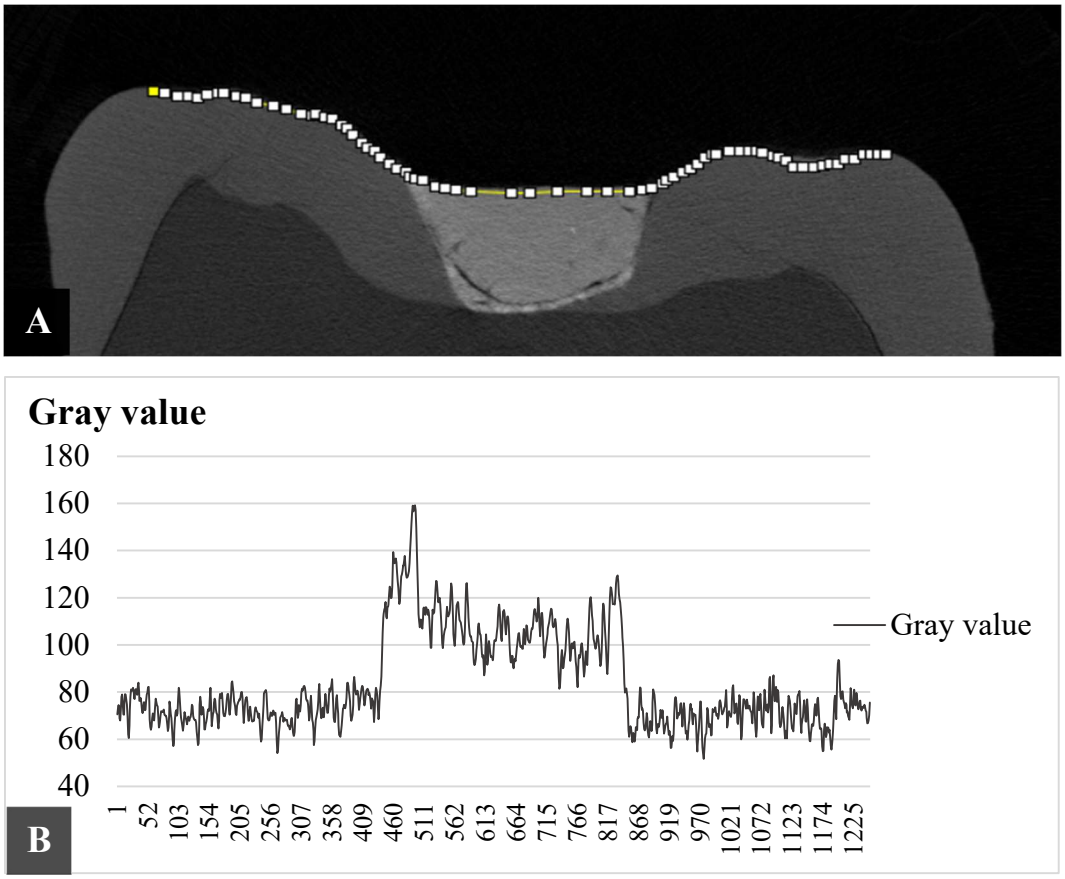

Figure 9. Line setting for plot profile on tooth specimen surface (A). Plot profile showed gray value on the line by pixel (B). as gray scale increased, shade on micro-CT image was shown brighter.

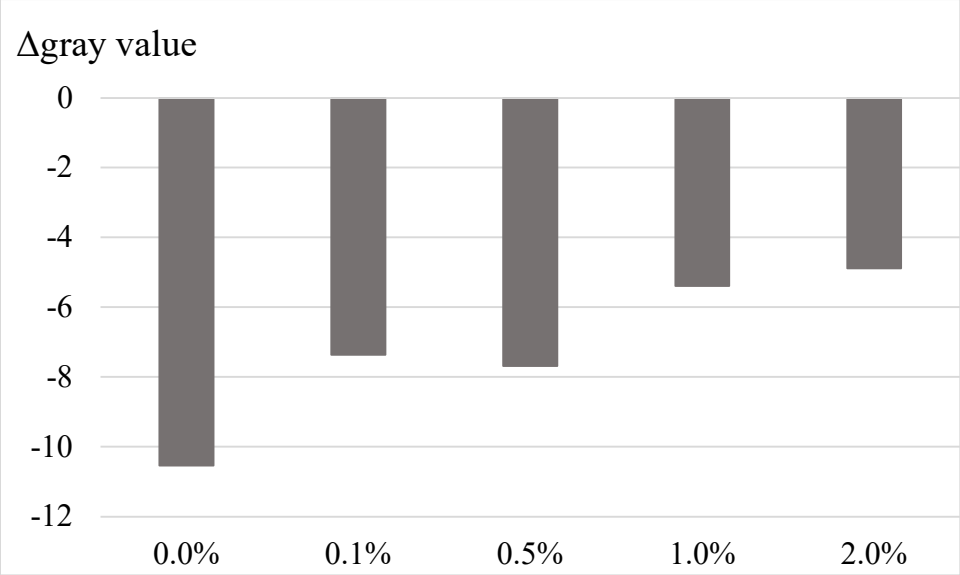

Figure 10. The difference between averaged gray value of bilateral enamel surface. 50 pixels from restoration margin were analyzed. . There was a tendency that the difference of gray value was decreased as UA concentration increased. (n=2)

IV. Discussion

S.mutans was reported to be the main causative factor of dental caries (Takahashi and Nyvad 2011). Thus, previous studies induced artificial caries using *S.mutans* to evaluate the anti-carious effect of UA (KIm, et al. 2011; Kim, et al. 2013). However, normal flora in oral cavity consist of not only *S.mutans* single species but very large number of unknown types of oral bacteria (Moore and Moore 1994; Paster, et al. 2001). The cariogenicity of dental biofilms is determined by interactions between the various bacterial species constituting the dental biofilm rather than by single species.

In vivo dental plaque biofilms consist of complex communities of oral bacteria that are a challenge to replicate *in vitro*. Because of the ethical issues and limited access of in vivo studies, a need for a laboratory model to allow investigations under controlled conditions (Filoche, et al. 2007; Wong and Sissons 2001). To minimize the difference of bacterial composition between *in vivo* and *in vitro*, in this experiment we used the human saliva inoculum to create dental microcosm biofilm. In present study, we designed to induce caries based on biofilm caries model for artificial caries (Filoche, et al. 2007; Lee, et al. 2013). With a one-time saliva inoculation, under culture conditions and daily renewal of the artificial saliva growth medium, present study was designed to replicate dental plaque biofilms that consist of complex communities of oral bacteria. Thus, the plaque biofilms that are generated from human saliva appear to reflect the complexity, diversity and heterogeneity of *in vivo* plaques (Sissons 1997; Wimpenny 1997).

According to bacterial analysis results, there was no *streptococcus mutans* group which was reported to be a main causative factor of dental caries (Takahashi and Nyvad 2011). It was agreed with microcosm dental biofilm model study(Filoche, et al. 2007), in which *streptococcus mutans* group also was not found in human saliva. Many other species appeared to be involved in dental caries other than *streptococcus mutans* (Becker, et al. 2002). Elevated levels of *S. salivarius*, *S. sobrinus*, and *S. parasanguinis* were also associated with caries. *Veillonella*, which metabolizes lactate, was associated with caries and was highly correlated with total acid producing species (Gross, et al. 2012). These bacteria were also found in present study and appeared to be alternative pathogens for artificial caries induction.

QLF was used for caries progression evaluation in this study. On QLF data, the absolute value of mean fluorescence loss (ΔF) and total fluorescence loss volume (ΔQ) increased after caries induction compared with the before caries induction. This means caries-related biofilms from human saliva inoculum induced demineralization on the tooth surface. The difference between before and after caries induction of ΔF and ΔQ decreased in the UA containing resin cement groups. This means that caries was more progressed in the non-UA containing resin cement group, and it was significant in the difference of ΔQ when at least 1.0% UA was included. Therefore, the null hypothesis that there was no difference in the caries progression in all experimental groups was rejected.

Because very small amount of fluorescence loss on enamel surface of tooth specimens could exist, which could not be detected on white light, there were many specimens that

showed negative value of ΔF and ΔQ before caries induction. Thus, it was quantitative difference that matters, rather than absolute value of ΔF and ΔQ before caries induction.

In this experiment, micro-CT was taken as a pilot study for the evaluation of the CT for the caries progression. On micro-CT images, gray value of enamel surface around restoration margin was also decreased compared to sound enamel surface. This means microcosm dental biofilms from human saliva inoculum induced demineralization on the tooth surface. And the difference of gray value between demineralized area around restoration and sound enamel surface decreased as UA concentration increased. it appeared that the UA in the resin cement, to some extent, inhibited caries progression and it was agreed with the results of QLF analysis. But because the sample count for micro-CT analysis was 2 for each group, statistical analysis could not be performed.

UA concentration enough to exert significant anti-carious effect was different from previous studies. While the significant concentration was 1.0% using dental microcosm biofilm in present study, on the other hand, the significant concentration was 0.5% in the previous study using *S.mutans* single species (Yoo, et al. 2019). In another previous study that UA was incorporated to composite resin matrix, the lower concentration of UA (0.1%) exhibited significant antibacterial effect (Kim, et al. 2013). Different significant concentrations might be attributed to the bacteria used in experiment or where into incorporate the UA, in other words, composite resin or resin cement. To establish the optimal concentration of UA, further studies are required to determine the optimal concentration and the addition method of UA.

Hydrophobic characteristics of ursolic acid may affect the long-term anti-bacterial activity. Some hydrophilic materials incorporated in composite resin were reported to readily diluted so that after several weeks, the concentration is no longer sufficient to exert any antibacterial effect (Hiraishi, et al. 2008). However, hydrophobic ursolic acid still showed significant inhibition effect against biofilm formation after 6 months storage in distilled water (Kim 2013). Hydrophobic ursolic acid could not leach out in the surrounding area so that ursolic acid could be in direct contact with the bacteria to exert its antibacterial effect. Although 6 months is not enough time to evaluate the long-term effect of ursolic acid, hydrophobic anti-bacterial materials are thought to exhibit longer anti-bacterial effect than hydrophilic materials. Further studies are needed to evaluate long-term effect.

V. Conclusions

Within the limitation of this study, artificial caries around restoration was less induced significantly in groups of resin cement containing UA more than or equal to 1.0% ($p<0.05$) in oral microcosm model. Resin cement containing UA as an antimicrobial agent might have potential to be used clinically at least 1.0% of UA concentration.

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Abstract (In Korean)

마이크로코즘 바이오필름을 이용한 우르솔릭산을 함유한 실험 레진 시멘트의 항우식 효과

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이차 우식은 복합 레진 수복의 주요한 실패 원인인 것은 많이 알려져 있고, 이것을 극복하기 위해 복합 레진에 항우식 효과를 가진 물질을 넣는 연구가 활발하다. 그 중에서도 우르솔릭산은 triterpenoids 의 일종으로 앞선 연구에서 *S.mutan*에 대해 우르솔릭산을 함유한 실험용 레진 시멘트가 항우식 효과가 있다고 밝혀졌다.

이에 본 연구에서는 우르솔릭산을 포함한 실험용 레진 시멘트를 이용하여 실제 임상과 유사한 조건의 구강 내 혼합균종에 대해서 항우식 효과가 있는지 입증하고, 항우식 효과를 위해 적절한 우르솔릭산의 농도를 알아보고자 한다.

5 가지 농도의(0, 0.1, 0.5, 1.0, 2.0 wt%) 우르솔릭산을 포함한 실험 레진 시멘트를 준비하고, 치아 시편은 발거된 인간의 대구치 50 개로 준비한다.

발거치의 교합면 상에 2mm x 4mm x 2mm (가로 x 세로 x 깊이) 크기로 와동을 형성하고 간접 수복용 레진 인레이를 제작하여 각 시편에 실험용 레진 시멘트로 접착하였다. 간접 수복용 레진 인레이는 Tescera^{TM} system (Body A1 shade, Bisco, Schaumberg, IL, USA)을 이용하였고 제조사의 지시대로 제작하였다. 그 후 수복물 주변 2mm 밖으로는 산 저항성 네일 바니쉬를 도포하여 수복물 주변의 치질에 대한 우식 정도만 평가하고자 하였다.

구강 내 혼합균종을 포함한 마이크로코즘 바이오필름을 유도하기 위해 24 시간 이상 구강 위생 관리를 하지 않은 건강한 성인 남성의 사람 타액을 모아 치아 시편에 접종하였다. 세균 배양 전 타액의 균종 분석을 시행한 결과 정상 세균총 범주의 균종들이 검출되었다. 치아 시편에 타액을 접종하기 전 특별한 오염의 증거가 없음을 확인하였다. 치아 시편에 타액 접종하고 37 ℃ 혐기 조건으로 4 시간 배양한 후, 타액은 흡인하여 제거하고 basal medium mucin (BMM) 와 0.5% 설탕이 혼합된 배양액을 첨가하였다. 배양액은 24 시간마다 교체하였고, 총 10 일 간 배양하여 치아 시편에 인공 우식을 유도하였다.

우식 전후로 정량적 광 유도 형광법 (QLF, Quantitative Light-induced Fluorescence)을 이용하여 우식 진행 정도를 비교하였다. 이후 통계분석을 통해 우르솔릭산의 농도에 따른 우식 유도 정도를 정량적으로 분석하여 우르솔릭산의 항우식 효과를 확인하고자 한다. 상기의 실험 결과는 일원 변량 분석 및 Tukey 사후 검정 통계 분석법으로 분석하였다.

QLF 지표인 ⊿F (-%) 와 ⊿Q (-% · Px) 값은 우르솔릭산 함유 농도가 증가함에 따라 우식 유도 후에 변화량이 감소하는 경향을 나타냈다. 이러한 경향은 ⊿F 보다는 ⊿Q 에서 더 뚜렷하게 나타났는데 대조군의 ⊿Q 의 우식 유도 전후의 변화량은 -3660.6 인데 반해, 2% 우르솔릭산 농도 그룹의 값은 -404.5 로 나타났다. 이는 우르솔릭산이 인공 우식 유도를 억제했다는 의미로 해석할 수 있다. ⊿F 에서는 우르솔릭산 함유 농도가 증가함에 따른 우식 유도 후의 변화량이 통계적으로 유의차가 없었던 반면, ⊿Q 에서는 통계적으로 유의차가 있었다 ($P<0.05$). 여기에서 1.0% 이상의 우르솔릭산을 함유한 레진 시멘트에서 인공 우식이 수복물 주변의 치질에서 통계적으로 유의하게 적게 유도되었다는 것을 알 수 있다 ($P<0.05$). 하지만 1.0% 이상의 우르솔릭산을 함유한 실험군 사이에 유의미한 통계적 유의차는 없었다.

본 연구의 결과에 의하면, 우르솔릭산을 함유한 레진 시멘트는 1.0% 이상의 우르솔릭산을 함유한 레진 시멘트에서 항우식 효과를 나타내었다.

핵심 되는 말 : 항우식성 레진 시멘트; 우르솔릭산; 마이크로코즘 바이오필름