The change of *Streptococcus mutans* biofilm inhibition effect of composite resins containing bioactive glass-ursolic acid after 6 month water storage

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The change of *Streptococcus mutans* biofilm inhibition effect of composite resins containing bioactive glass-ursolic acid after 6 month water storage

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감사의 글

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실험 기간 내내 항상 큰 도움을 준 Zou yunyun 선생님과 서울대학교 안석준 교수님, 서울대학교 교정과 실험실 선생님들께도 큰 감사를 드립니다. 또한 어려운 부탁에도 흔쾌히 도움 주신 서울대학교 강대용 교수님께 진심으로 감사드립니다. 힘들고 어려울 때마다 격려를 아끼지 않고 힘이 되어준 보존과 동기 김현 선생님께도 고마움 전합니다. 마지막으로 항상 저를 다독여주고 할 수 있다는 믿음을 심어준 가족들에게 감사를 전합니다.

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Abstract

The change of *Streptococcus mutans* biofilm inhibition effect of composite resins containing bioactive glass-ursolic acid after 6 month water storage

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(Directed by Prof. Jeong-won Park, D.D.S., M.S., Ph.D.)

1. Objective

The aim of this study was to investigate the *S.mutans* biofilm formation on composite surface containing bioactive glass and ursolic acid when exposed to distilled water for 6 months compared with fresh antibacterial composite resin specimen.

2. Materials and methods

Four experimental groups and one control group were prepared : Conventional composite (10%wt OX50 silica nanofiller instead of BAG) as control, BAG filler group (BAG), BAG filler coated with UA group (UA BAG), UA added to resin matrix (BAG+UA Monomer), and UA added to BAG and resin matrix (UA BAG+UA Monomer). All specimens were stored in distilled water for 6 months (the aged resin group). After

sterilization with ethylene oxide gas, biofilm assay was performed again on all five groups. The specimens for the aged resin group were fabricated on the previous study by Kim (Kim B, 2013), and re-used for this study.

For biofilm assay, *S. mutans* was incubated for 24 hours with each composite resin disk specimen in a biofilm medium with either glucose or sucrose in the presence or absence of a salivary coating. The adherent bacteria were quantified after sonication of the specimen by counting the colony forming units of viable bacteria.

Calcium and fluoride ions concentration released from new and aged specimens were analyzed quantitatively by using Ion Chromatography (IC).

Five disk specimens of each group were immersed and stored in 5 ml distilled water for 24 hours. The measurement of ion release was carried out by using the IC on 1-day interval for 7 days.

The t-test for variable CFU was used for biofilm assay to analyze statistical significance between new and aged groups. To analyze the effect of composite composition on the biofilm formation, one-way analysis of variance (ANOVA) followed by a multiple-comparison Tukey test was performed. The amount of calcium and fluoride ion release between old resin groups and new resin groups was compared using t- test at a 5% level of significance.

3. Result

When glucose was given as a carbohydrate source, there was no significant difference between new and aged resin under saliva non-coating condition. In new resins, the CFU values of all experimental groups were significantly lower than control group. However, in aged resin, only the BAG + UA Monomer group showed significantly lower CFU value than control group. In saliva coating condition, the CFU value of all aged resin groups significantly increased compared to all new resin groups. In the new resin groups, the CFU

value of all experimental groups was significantly lower than control. However, there was no significant difference between control and BAG group on aged resin.

When sucrose was given as a carbohydrate source, under salivary non-coating condition, CFU value of aged resin significantly increased more than new resin on all experimental groups except in the control group. In the new resin groups, the CFU value of BAG + UA Monomer, UA BAG + UA Monomer group was significantly lower than control. However, there was no significant difference between all groups in aged resin groups. In saliva coating condition, the CFU value of BAG + UA monomer, UA BAG + UA Monomer group was significantly lower than control group in aged resin and new resin.

On both calcium and fluoride, new resin showed higher amount of ion release than aged resin. A significant difference was shown on fluoride ion release all day, and new resin showed significantly higher release of calcium ion on the 1^{st}, 5^{th} day. On both calcium and fluoride, a significantly high concentration of ion release was shown on the 1^{st} day in new resin specimen. The amount of ion release showed significant decrease on the 2^{nd} day, but increased on the 3^{rd} day.

4. Conclusion

In glucose source, experimental new composites containing BAG and/or UA showed significant reduction of biofilm formation by S. *mutans*. However, after storage in distilled water for 6 months, experimental composites containing BAG showed decreased biofilm inhibition effect. The composites with UA added to the monomer still showed significant inhibition effect of the biofilm formation by *S. mutans* even after storage in distilled water. In sucrose source, the new composites of UA Monomer group showed significant antibacterial effect under any salivary treatment. After storage in distilled water for 6 months, the biofilm formation was affected by salivary treatment. In Saliva non-coating groups, there were no significant difference in all groups, and in saliva coating groups, BAG

+ UA Monomer and UA BAG + UA Monomer groups showed lower CFU values.

Following the results of this experiment, it can be concluded that the UA incorporated in monomer was more effective method to keep the antibacterial effect in any biofilm formation condition after 6 month water storage condition.

Within the limitation of this experiment, this result indicates that UA inhibits biofilm formation by *S. mutans* and suggests that UA has potential for use as an effective antibacterial agent to prevent dental caries in the future.

Key words : bioactive glass, ursolic acid, antibacterial composite resin, biofilm, *Streptococcus mutans,* water storage

The change of *Streptococcus mutans* biofilm inhibition effect of composite resins containing bioactive glass-ursolic acid after 6 month water storage

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Soomin Kim

I. Introduction

Dental caries is one of the most prevalent chronic diseases (Selwitz et al., 2007), and the composite resins have been used widely for treatment of this disease. *Streptococcus mutans* is a well-known major pathogen of this disease (Mitchell, 2003), and when it forms biofilm on the tooth and restoration surface, recurrent caries can be developed. Therefore, to control recurrent dental caries, it is necessary to inhibit the formation of cariogenic bacterial biofilm.

A number of studies have tried to develop antibacterial composite resins, using chlorhexidine diacetate (CHXA) (Hiraishi et al., 2008; Leung et al., 2005), sliver

nanoparticles (Cheng et al., 2012), 12-methacryloyloxydodecylpyridinium bromide (MDPB) (Imazato et al., 1997), quaternary ammonium polyethylenimine (PEI) nanoparticles (Beyth et al., 2006), and calcium phosphate (Melo et al., 2013) for inhibiting bacterial biofilms. However, these experimental antibacterial composite resins had several limitations, for examples, short-term effectiveness, decreased physical property, toxicity, or poor color stability (Fan et al., 2011; Kohnen and Jansen, 1995; Nohr and Macdonald, 1994), and none of them were successfully used in the clinic.

Bioactive glasses (BAG) have been used as scaffolds for bone tissue engineering (Hench et al., 2004; Rezwan et al., 2006; Xynos et al., 2001), and recently also used for treatment of hypersensitive teeth (Lynch et al., 2012). Bioactive glasses contain oxides of calcium, sodium, phosphorus, and silicon in a proportion that provides the material with surface activity (Stoor et al., 1998). Recently, it was observed that Bioactive glass and other silicate-based glasses have antibacterial (Allan et al., 2001; Hu et al., 2009; Lepparanta et al., 2008; Stoor et al., 1998) and anti-inflammatory effects (Day and Boccaccini, 2005).

Stoor et al. (1998) confirmed that the bioactive glass S53P4 (SiO_2 53%, Na_2O 23%, CaO 20%, and P_2O_5 4%) in aqueous solutions appears to have a broad antimicrobial effect on microorganisms of both supra- and subgingival plaque (Stoor et al., 1998). But in most studies, BAGs have been used in powder form, so it is necessary to observe the antimicrobial effect when it is added to restorative material. The antibacterial effect of composite resins containing BAG has not been reported enough.

Ursolic acid (UA) is one of the triterpenoid compounds that is isolated from edible and medicinal plants and has many beneficial effects, such as preventing liver damage, reducing inflammation, inhibiting tumor growth and reducing hyperlipidemia (Zhou et al., 2012). In addition, UA are relatively non-toxic (Liu, 1995). Some studies have confirmed that triterpenoid compounds have antibacterial activity (Fontanay et al., 2008; Scalon Cunha et

al., 2007; Zhou et al., 2012). Zhou et al. (2012) evaluated the antibacterial activity of triterpenoic acids (ursolic acid and oleanolic acid) against cariogenic microorganisms in vitro. They demonstrated that ursolic acid and oleanolic acid can reduce bacterial biofilm formation at 1/4 MIC, whereas higher UA concentrations display antibacterial activity against *S. mutans* in mature biofilms (Zhou et al., 2012).

In the previous studies reported that antibacterial composite resin containing BAG and UA have inhibitory effect on biofilm formation of *S. mutans* (Kim, 2013; Kim et al., 2013; Kim, 2012). UA was found to suppress the bacterial growth of *S. mutan*s when they were added to the matrix of commercial nanofilled composite (Kim et al., 2013). Another study evaluated whether UA has more antibacterial effect when it is incorporated to composites into BAG filler or matrix. Their study showed that antibacterial effect is largest when composites contain BAG filler and UA in the matrix group (Kim, 2013).

However, previous studies were performed right after production of fresh antibacterial composite resin specimen, within less than 24 hours. Therefore, in the previous research, we can conclude that BAG and UA suppress the bacterial growth of *S. mutan*s when they are added to the composite in the early period. However, the antibacterial effect can be changed by the sites of incorporation and mechanism if they were exposed in the aqueous condition, such as intraoral environment, and it is necessary to evaluate their long-term effect.

The objective of this study was to compare the antibacterial effect of different composites containing BAG and/or UA in fresh and 6-month aged composites, and which incorporation sites are more effective to keep their effect in aqueous condition.

II. Materials and Methods

1. Preparation of experimental composite resins

Antibacterial composites containing bioactive glass (BAG) filler and ursolic acid (UA) were prepared. BAG (62 mol% SiO_2, 31 mol% CaO, 4 mol% P_2O_5, and 3 mol% F) was manufactured by sol-gel methods, ball milled, sieved, and micronized (Sturtevant, Hanover, MA, USA). Average particle size was determined by laser particle size measurements and it was ranged from 0.04 to 3.0 μm (Beckman Coulter LS13 320, Brea, CA, USA) (Brown et al., 2011).

For making UA coated BAG (UA BAG), UA (U6753, Sigma Aldrich, St. Louis, MO, U.S.A.) was dissolved in 70% ethanol and mixed with BAG. Then, the solvent was evaporated under negative pressure in vacuum condition and complete evaporation was confirmed by comparing the weight before and after the treatment. To incorporate the ursolic acid into the resin matrix (UA Monomer), the ursolic acid was mixed with TEGDMA and stirred thoroughly with magnetic stirrer. Afterwards, BisGMA was added in 50:50 ratios to the resin matrix.

For this experiment, one control and four experimental composites were prepared following the composition of Table 1: Conventional composite (10% OX50 silica nanofiller instead of BAG) as control, 10% BAG filler containing group (BAG), 10% BAG filler coated with UA containing group (UA BAG), 10% BAG filler and 0.5% UA containing matrix group (BAG+UA Monomer), and 10% BAG filler coated with UA(0.25%) and 0.25% UA containing matrix group (UA BAG+UA Monomer).

All specimens were prepared in uniform shape and size (5 mm in diameter, 2 mm in height) because the same Teflon mold were placed between two glass slides on both sides, and

light curing was performed on composite resins in mold for 40 seconds. Since the biofilm had been formed at the upper surface, extra attention was required not to turn the specimen upside down. Thus the bottom side was marked with an oil-based pen. The upper surfaces of specimens were polished with 800-grit, 1200-grit, 1500-grit SiC papers (Deerfos, Inchon, Korea) in order. The specimens for the aged resin group (stored in distilled water) were fabricated on the previous study by Kim (Kim, 2013), and re-used for this study. After biofilm assay on previous study (Kim, 2013), all specimens were performed ultrasonic cleaning and stored in distilled water for 6 months. Distilled water was replaced with new distilled water once a week. After 6 months, specimens were sterilized with ethylene oxide gas.

To assure that this procedure can remove the previously formed biofilm, scanning electron microscopic (300M, Hitachi, Tokyo, Japan) images were taken.

Table 1. Filler and matrix compositions of experimental groups

Groups	Filler (%)				Matrix (%)
	Glass	OX50	BAG	UA	
Control	61	10	0	0	29
BAG	61	0	10	0	29
UA BAG	61	0	9.5	0.5	29
BAG + UA Monomer	61	0	10	0	29 (0.5% UA included)
UA BAG + UA Monomer	61	0	9.75	0.25	29 (0.25% UA included)

The composition of matrix used in this study was 49.38% of Bis-GMA, 49.38% of TEGDMA, 0.40% of CQ, 0.80% of EDMAB, and 0.05% of MEHQ.

Control : silica nanofillers

BAG: bioactive glass

UA : ursolic acid

Bis-GMA: bisphenol A diglycidyl methacrylate

TEGDMA: triethylene glycol dimethacrylate

CQ: camphorquinone

EDMAB: amine-ethyl-4-dimethylaminobenzoate

MEHQ: monoethyl ether hydroquinone

UA BAG group : Ursolic acid was coated to BAG fillers

BAG + UA Monomer group : Ursolic acid was dissolved in resin matrix

UA BAG + UA Monomer group : Ursolic acid was added both BAG filler and resin matrix

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2. Biofilm assay

2.1. Assignment of the experimental groups

Four experimental composite resins (BAG, UA BAG, BAG+UA Monomer, UA BAG+ UA Monomer) were compared with the control group, and comparison between aged resin group and new resin group was performed. To assess the effect of saliva, the composite resin disks were submerged in either phosphate buffered saline (PBS, pH = 7.2 : non-coating group) or unstimulated whole saliva (UWS: saliva coating group), and were placed on a rocking incubator for 2 hours. For the nutrient source provided for bacterial growth, either glucose or sucrose was added to the medium. The combinations of all these variables are displayed in Figure 1.

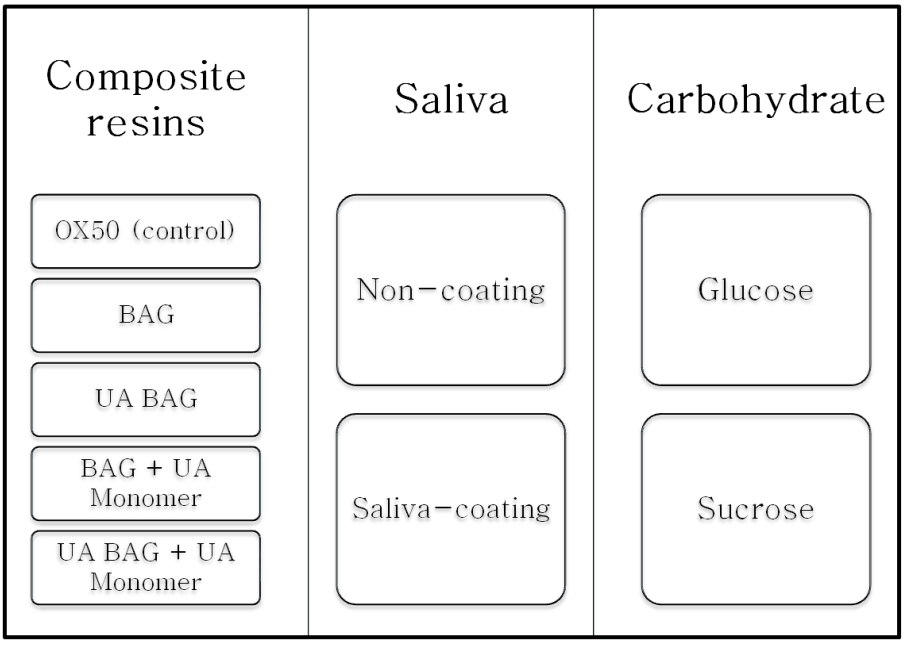


Fig. 1. Assignment of the experimental groups. Five different composites were tested with different salivary coating and carbohydrate sources.

BAG: bioactive glass, UA: ursolic acid.

2.2. Preparation of *Streptococcus mutans* and saliva

UWS was collected from four healthy volunteers by the spitting method. All participants had undergone dental examination prior to the experiment to ensure that they were free from any acute caries or periodontal disease. Saliva was collected between 9:00 a.m. to 11:00 a.m. to minimize the effects of diurnal variability on salivary composition (Hardt et al., 2005). The collected samples were centrifuged at 3,500 g for 10 minutes to remove any cellular debris. The resulting supernatant was then filter-sterilized through a Stericup & Steritop (Millipore, Billerica, MA, USA), and stored in 4℃ before use.

S. mutans UA159 was grown in the brain heart infusion (BHI) agar plate. A colony of *S. mutans* was transferred to BHI broth, and the broth was incubated overnight. The culture was then re-suspended to BHI broth in 1 : 20 ratio and incubated again until it reached exponential phase. The optical density at 600 nm (OD_{600}) was measured, and the broth was used when the OD_{600} reached 0.5 (approximately 6.5 x 10^7 CFU per mL).

2.3. Biofilm assay

The sterilized resin disks were transferred to a polystyrene 24-well (flat bottom) cell culture cluster (Corning Inc., Corning, City NY, USA). The prepared *S. mutans* suspension was diluted to BHI broth which was kept warm in the incubator. The medium contained 20 mM of either glucose or sucrose as a carbohydrate source. The composite resin disks which were treated with either UWS or PBS were inoculated with 1 mL medium containing 1:100 dilution of *S. mutans* suspension. Biofilms were allowed to form at 37℃ in a 5% CO_2 for 24 hours.

Afterwards, the disks were washed twice with 2 mL of sterile PBS to remove planktonic and loosely bound cells. The specimen was then placed in a conical tube with 3 ml of PBS and sonicated using 30 seconds pulse at 20 W four times with simultaneous cooling by

placing the tube in the ice box. The disrupted biofilm suspension was serially diluted, plated in duplicate on BHI agar, and incubated at 37℃ in a 5% CO2 atmosphere for 48 hours. The plating was carried out by automatic sample plater (easySpiral®, Interscience, Saint Nom, France). The accuracy of dilution and plating by easySpiral® apparatus was confirmed by previous study (Kim, 2013). After 48 hours, colony forming units (CFUs) were counted visually, scaled by dilution factors. The colonies that did not grow symmetrically were excluded from counting.

If the CFU values between duplicates differed more than 20%, the data was discarded. For statistical reason, all data acquired on the same day were also discarded, too. The flow chart of procedures was summarized in Figure 2.

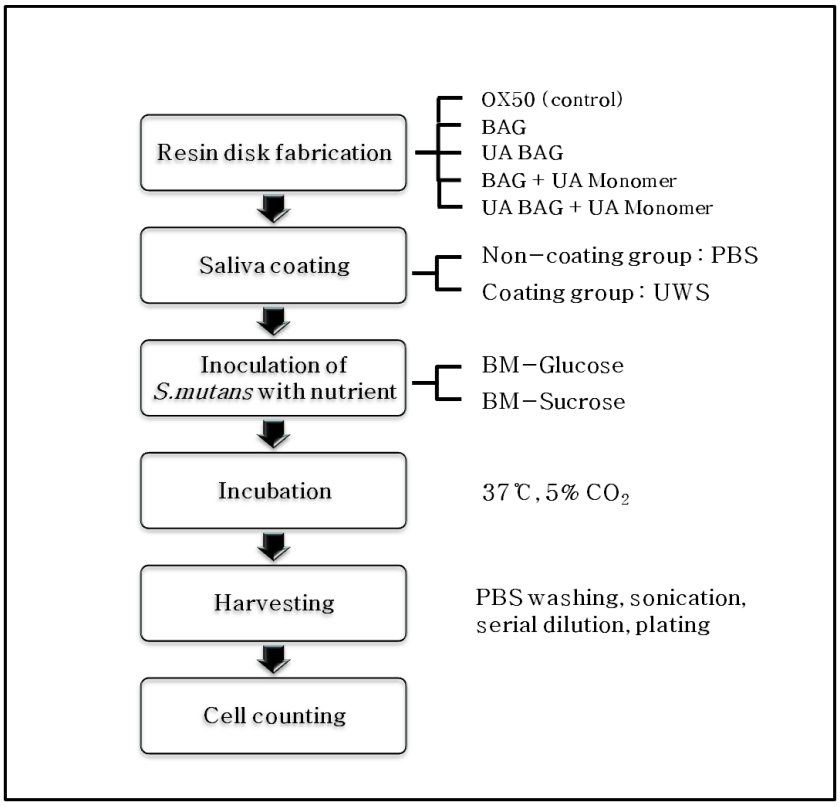


Fig. 2. Procedures of biofilm assay.

PBS : phosphate-buffered saline, UWS : unstimulated whole saliva,

BM : biofilm medium, BAG: bioactive glass, UA: ursolic acid.

3. Ion release measurement using ion chromatography

3.1. Preparation of the specimens

Calcium and fluoride ions concentration released from new and aged specimens were analyzed quantitatively by using Ion Chromatography(IC) (790 Personal IC, Metrohm, Herisau, Switzerland).

Five disk specimens of each group were immersed and stored in individual plastic containers with 5 ml distilled water for 24 hours. At the time of ion release measurement, each specimen was removed from its container and the storage solution was extracted using a 5 cc syringe for analysis. Then the specimen was transferred to a new container with 5 ml of fresh distilled water, and this storage transfer method was continued for 7 days on 1-day interval. Free calcium and fluoride ions showed well-defined retention time and the peak corresponding to ion concentration could readily be determined from the chromatogram. The ion release of IC can be measured up to 0.001ppm.

4. Statistical analysis

The t-test for variable CFU was used for biofilm assay to analyze statistical significance between new and aged groups. To evaluate the effect of different storage time (new vs. aged) and composition of composite resins, two-way analysis of variance was performed. To analyze the effect of composite composition on the biofilm formation, one-way analysis of variance (ANOVA) followed by a multiple-comparison Tukey test was performed. The amount of calcium and fluoride ions from new and aged composites was compared using t-test with adjusted P value considering repeated measurement during 7

days. The P value less than 0.05 was considered to be statistically significant (SAS 9.3 ver., SAS Institute Inc., Cary, NC).

III. Results

1. Biofilm assay

The 2-way ANOVA demonstrated that there was no interaction between the type of composite resins and storage period in distilled water under any carbohydrate source and salivary treatment. Also, CFU values according to type of composite resins and storage period showed significant difference regardless of carbohydrate source and salivary condition ($p < 0.05$).

1.1 The influence of the storage period in distilled water

1.1.1. Glucose used as carbohydrate source

In Table 2 and Figure 3, there was no significant difference between new and aged resin under saliva non-coating condition. However, in saliva coating condition, the CFU value of all aged resin groups significantly increased compared to the new resin groups ($p < 0.05$).

1.1.2. Sucrose used as carbohydrate source

In Table 3 and Figure 4, in salivary non-coating condition, CFU value of aged resin significantly increased more than new resin on all experimental groups except in the control group ($p < 0.05$). However, in saliva coating condition, the CFU value of aged resin was significantly lower than new resin in control, BAG and UA BAG groups ($p < 0.05$).

1.2. The influence of the type of composite resins

1.2.1. Glucose used as carbohydrate source

In saliva non-coating condition, only the BAG + UA Monomer group showed significantly lower CFU value than control group in aged resin ($p < 0.05$). However in new resins, the CFU values of all experimental groups were significantly lower than control group ($p < 0.05$).

In saliva coating condition, there was no significant difference between control and BAG group on aged resin. However, the CFU value of UA BAG, BAG + UA Monomer, and UA BAG + UA Monomer group was significantly lower than control group ($p < 0.05$). There was no significant difference among experimental groups. In the new resin groups, the CFU value of all experimental groups was significantly lower than control ($p < 0.05$). Also, there was no significant difference among experimental groups.

1.2.2. Sucrose used as carbohydrate source

In Saliva non-coating condition, there was no significant difference between all groups of aged resins. In the new resin groups, the CFU value of BAG + UA Monomer, UA BAG + UA Monomer group was significantly lower than control ($p < 0.05$), but no significant difference could be seen between all experimental groups.

In saliva coating condition, the CFU value of BAG + UA monomer, UA BAG + UA Monomer group was significantly lower than control group in aged resin and new resin ($p < 0.05$), but there was no significant difference between all experimental groups.

Table 2. Biofilm formation by *S.mutans* on various experimental groups in the presence of glucose. The amount of bacteria are expressed as CFU/ml (mean ± S.D.)

	Group / Saliva	OX	BAG	BAG UA	UA Monomer +BAG	UA Monomer +BAG UA
New resin	Saliva non-coating	6.01×10^{7} $\pm1.80\times10^{7}$	3.95×10^{7} $\pm1.02\times10^{7}$	3.90×10^{7} $\pm1.37\times10^{7}$	2.52×10^{7} $\pm1.03\times10^{7}$	3.28×10^{7} $\pm1.27\times10^{7}$
	Saliva coating	1.71×10^{6} $\pm6.92\times10^{5}$	1.01×10^{6} $\pm3.92\times10^{5}$	9.41×10^{5} $\pm2.96\times10^{5}$	6.12×10^{5} $\pm2.67\times10^{5}$	6.87×10^{5} $\pm2.95\times10^{5}$
Aged resin	Saliva non-coating	6.06×10^{7} $\pm3.63\times10^{7}$	4.34×10^{7} $\pm2.62\times10^{7}$	4.31×10^{7} $\pm2.85\times10^{7}$	2.53×10^{7} $\pm1.61\times10^{7}$	3.36×10^{7} $\pm2.04\times10^{7}$
	Saliva coating	2.53×10^{6} $\pm1.05\times10^{6}$	1.68×10^{6} $\pm9.13\times10^{5}$	1.63×10^{6} $\pm5.19\times10^{5}$	1.05×10^{6} $\pm5.33\times10^{5}$	1.27×10^{6} $\pm6.77\times10^{5}$

Table 3. Biofilm formation by *S.mutans* on various experimental groups in the presence of sucrose. The amount of bacteria are expressed as CFU/ml (mean ± S.D.)

	Group / Saliva	OX	BAG	BAG UA	UA Monomer +BAG	UA Monomer +BAG UA
New resin	Saliva non-coating	2.16×10^{7} $\pm1.23\times10^{7}$	1.20×10^{7} $\pm9.10\times10^{6}$	1.19×10^{7} $\pm9.01\times10^{6}$	7.63×10^{6} $\pm7.46\times10^{6}$	8.41×10^{6} $\pm8.22\times10^{6}$
	Saliva coating	2.94×10^{7} $\pm9.19\times10^{6}$	1.89×10^{7} $\pm8.84\times10^{6}$	1.82×10^{7} $\pm7.48\times10^{6}$	1.30×10^{7} $\pm6.78\times10^{6}$	1.43×10^{7} $\pm7.17\times10^{6}$
Aged resin	Saliva non-coating	2.66×10^{7} $\pm6.35\times10^{6}$	2.14×10^{7} $\pm6.04\times10^{6}$	2.12×10^{7} $\pm5.24\times10^{6}$	1.97×10^{7} $\pm9.61\times10^{6}$	2.00×10^{7} $\pm8.02\times10^{6}$
	Saliva coating	1.84×10^{7} $\pm8.51\times10^{6}$	1.21×10^{7} $\pm3.87\times10^{6}$	1.18×10^{7} $\pm5.25\times10^{6}$	9.28×10^{6} $\pm4.59\times10^{6}$	9.25×10^{6} $\pm6.52\times10^{6}$

Table 4. Relative ratio (CFU mean value in experimental group/CFU mean value in control group, %) of antibacterial effect in the presence of glucose. Ratio of control group is expressed as 100%.

		OX	BAG	BAG UA	BAG + UA Monomer	UA BAG + UA Monomer
Saliva non-coating	New resin	100.00	65.72	64.89	41.93	54.58
	Aged resin	100.00	71.62	71.12	41.75	55.45
Saliva coating	New resin	100.00	59.06	55.03	35.79	40.18
	Aged resin	100.00	66.40	64.43	41.50	50.20

Table 5. Relative ratio (CFU mean value in experimental group/CFU mean value in control group, %) of antibacterial effect in the presence of sucrose. Ratio of control group is expressed as 100%.

		OX	BAG	BAG UA	BAG + UA Monomer	UA BAG + UA Monomer
Saliva non-coating	New resin	100.00	55.56	55.09	35.32	38.94
	Aged resin	100.00	80.45	79.70	74.06	75.19
Saliva coating	New resin	100.00	64.29	61.90	44.22	48.64
	Aged resin	100.00	65.76	64.13	50.43	50.27

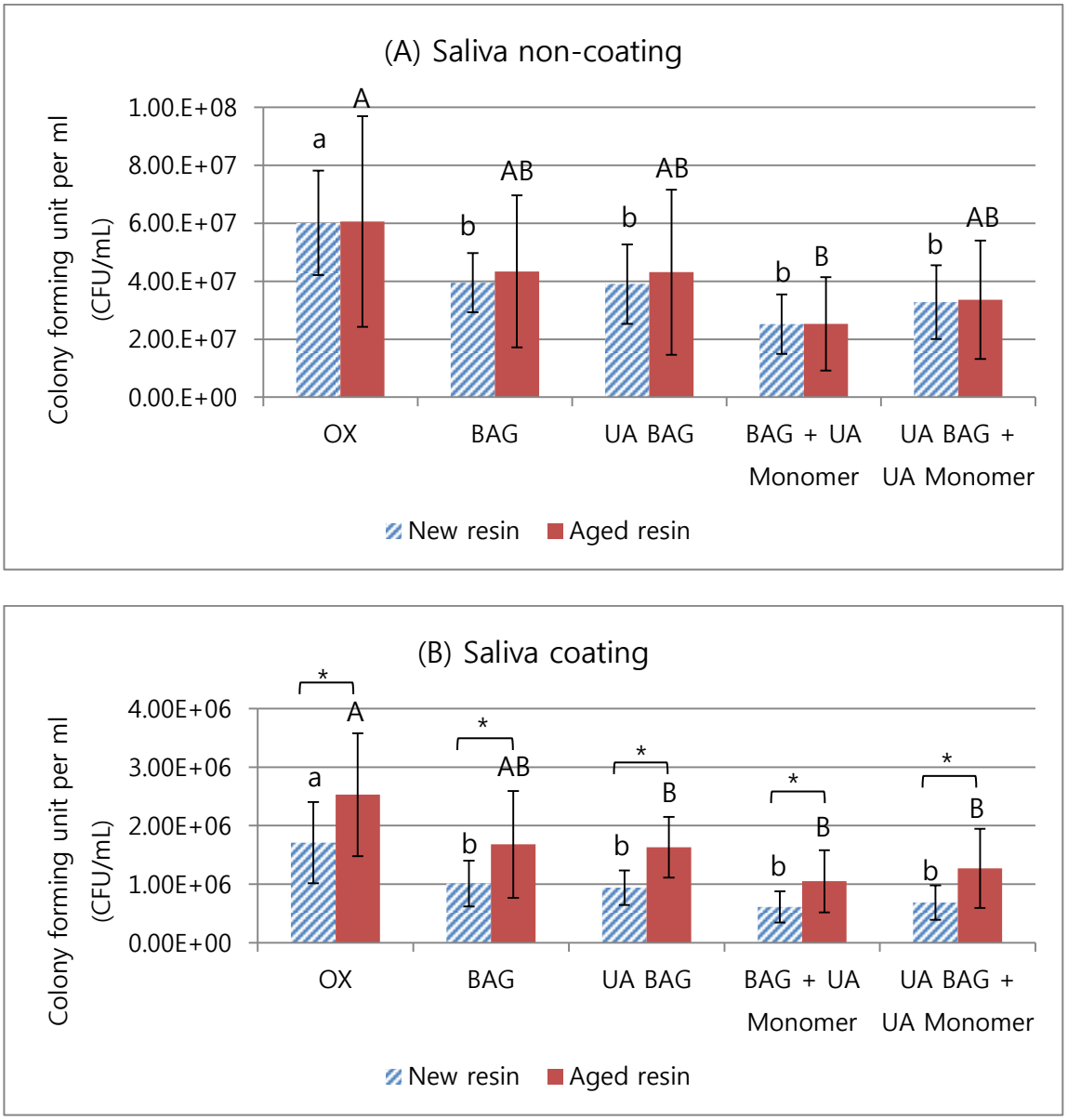


Fig.3. Biofilm formation by *S. mutans* on various experimental composite resins in BM glucose. (A) CFU per ml on saliva non-coating treatment. (B) CFU per ml on saliva coating treatment. Different letter indicates statistically significant differences between groups according to resin type ($P < 0.05$) (small letter : significant differences in new resin, capital letter : significant differences in aged resin). Significant differences ($p < 0.05$) between new resin and aged resin are indicated by asterisk.

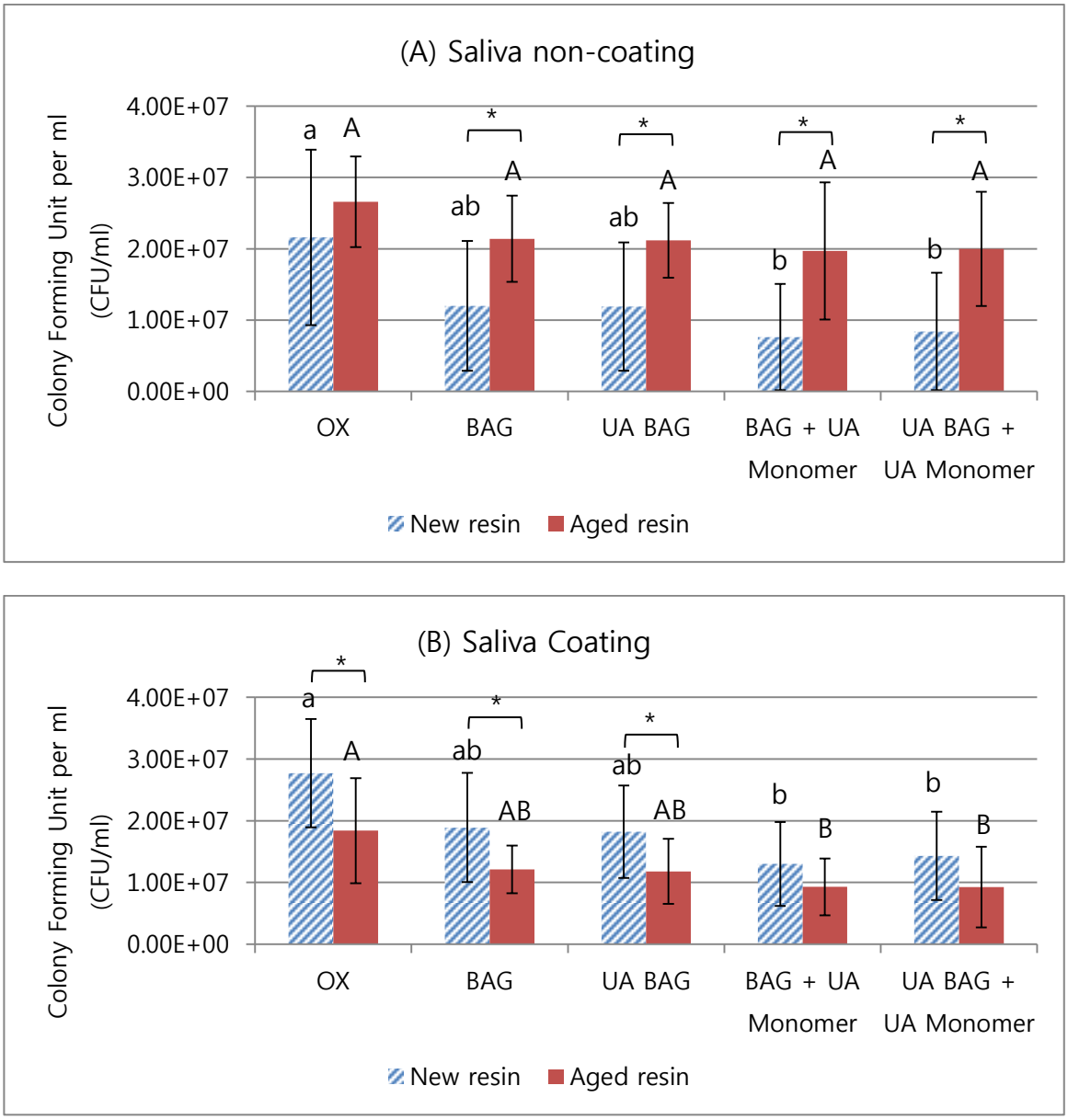


Fig. 4. Biofilm formation by *S. mutans* on various experimental composite resins in BM sucrose. (A) CFU per ml on saliva non-coating treatment. (B) CFU per ml on saliva coating treatment. Different letter indicates statistically significant differences between groups according to resin type ($P < 0.05$) (small letter : significant differences in new resin, capital letter : significant differences in aged resin). Significant differences ($p < 0.05$) between new resin and aged resin are indicated by asterisk.

2. Ions release measurement using ion chromatography

2.1. The influence of the type of composite resins (aged resin group and new resin group)

The data comparing calcium and fluoride ions release in aged resin group and new resin group during experimental period in distilled water is shown as a Table 6, 7 and a graph in Fig. 5.

2.1.1. Calcium ion release

The amount of calcium ion release on new resin group was observed to be significantly higher than aged resin group on day 1, 5 ($p < 0.05$), not on day 2, 3, 4, 6, 7 (Fig. 5(A)).

2.1.2. Fluoride ion release

The amount of fluoride ion release on new resin group was observed to be significantly higher than aged resin group on all experiment period ($p < 0.05$)(Fig. 5(B)).

2.2. The influence of the storage time of composite resins

The mean value of calcium and fluoride ion released into distilled water is presented in Table 6, 7. In all control groups, calcium and fluoride ions were not detected.

2.2.1. Calcium ion release

In new resin specimen, a significantly high concentration of ion release was shown on the 1^{st} day. The amount of ion release showed significant decrease on the 2^{nd} day, but

increased on the 3rd day. From the 3rd to the 7th day, the ion release amount was not changed a lot.

The release of calcium ions in aged resin specimen was similar from the 1st to the 7th day.

2.2.2. Fluoride ion release

The amount of fluoride ion release from new resin specimens was highest on the 1st day. The ion release steeply decreased on the 2nd day, just as shown in the calcium ion, the amount increased on the 3rd day. The fluoride ion release showed tendency to decrease from the 5th to the 7th day.

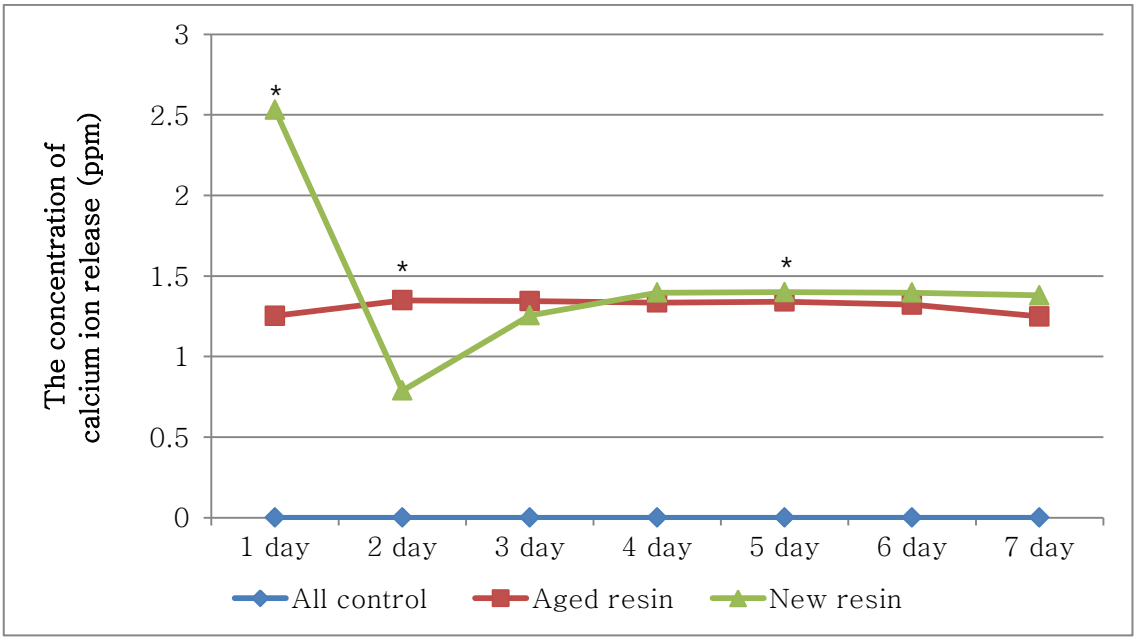
The amount of ion released from aged resin specimens was shown to be minimal on the 1st day, and stopped release completely starting from the 2nd day (ppm value=0).

Table 6. The amount of calcium ion release according to storage time on various experimental group. The concentrations of calcium ion are expressed in ppm. (mean value)

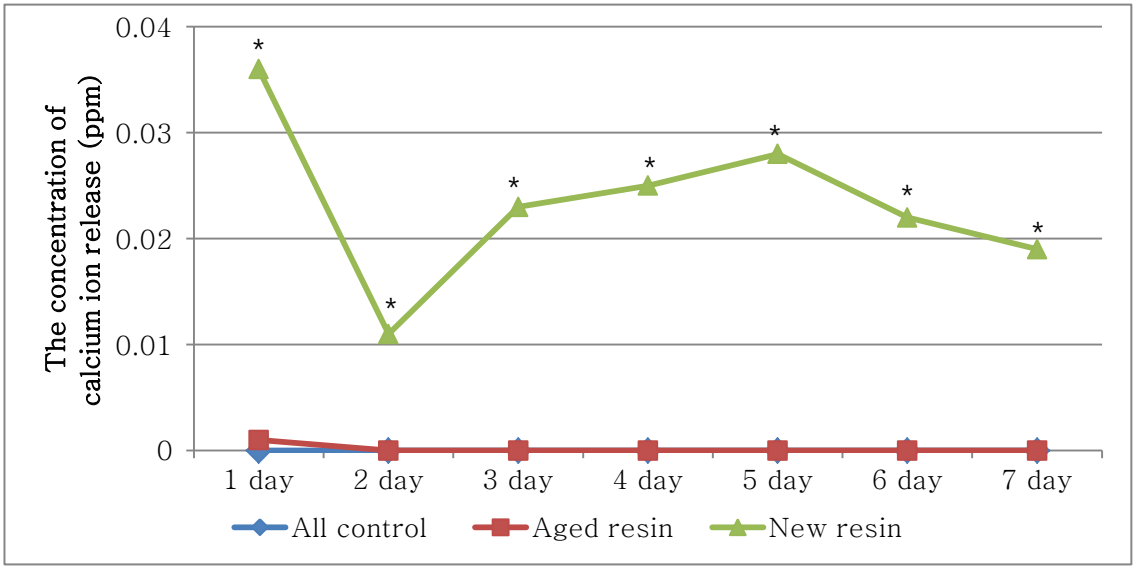
	1 day	2 day	3 day	4 day	5 day	6 day	7 day
New resin	2.532	0.789	1.256	1.397	1.400	1.397	1.380
Aged resin	1.253	1.349	1.344	1.335	1.341	1.322	1.249

Table 7. The amount of fluoride ion release according to storage time on various experimental group. The concentrations of fluoride ion are expressed in ppm. (mean value)

	1 day	2 day	3 day	4 day	5 day	6 day	7 day
New resin	0.036	0.011	0.023	0.025	0.028	0.022	0.019
Aged resin	0.001	0.000	0.000	0.000	0.000	0.000	0.000



(A) The concentration of calcium ion release according to storage time



(B) The concentration of fluoride ion release according to storage time

Fig. 5. The concentration of calcium and fluoride ion release per day on aged resin group and new resin group (ppm). Significant differences (adjusted p value <0.05) between new resin and aged resin are indicated by asterisk. (adjusted p-value : p-value considering repeated measurement during 7 days)

IV. Discussion

There have been numerous studies investigating antibacterial effect of composite resins containing various antibacterial agents in the past. However, they all had a common limitation of focusing only on the short-term effectiveness of the antibacterial agents. The longevity of dental restorations is often determined by their ability to resist plaque formation and consequently avoid secondary caries (Montanaro et al., 2004; Unosson et al., 2012). Although it has been suggested that early plaque formation on solid surfaces is influenced predominantly by the oral environment rather than by material-dependent parameters (Hannig, 1999), composite resins having antibacterial properties could successfully prevent or delay caries formation and prolong the longevity of restorations. This study assessed the long-term antibacterial effect of composite resins containing BAG and UA on *S. mutans* biofilm formation after 6 months storage in distilled water.

When we compared the antibacterial effect of the new and aged resins, some aged composite showed decreased antibacterial effect but some did not - for example, sucrose saliva coating and glucose saliva non-coating groups (Table 4 and 5). The possible reason why the aged groups showed decreased activity could be explained by the surface-free energy change in composite resin during distilled water storage (Tanner et al., 2001). After water storage, the contact angle of materials with high surface-free energy decreases, resulting in the increase of surface wettability. On a high surface-free energy substrate such as glass, increase in surface wettability and surface-free energy may produce more favorable condition to adhesion in terms of surface area and chemical reactivity.

However, when sucrose was used as carbohydrate source under saliva coating, the new resin showed greater decrease of CFU value than aged resin. When sucrose is applied as carbohydrate source, *S. mutans* forms insoluble glucan under saliva coating condition, leading to promotion of biofilm formation. Due to this phenomenon, in the new resin group, there was higher CFU value under saliva coating condition than under non-coating condition. The lower CFU value in the aged resin group may be due to the occurrence of change in surface energy under saliva condition after water storage, leading to the offset of the effect of glucan. However, this is only an assumption, so further study is necessary.

It is difficult to conclude the changes in antibacterial properties of composite resin after 6-month storage in distilled water in one sentence, due to the fact that the significant difference in CFU value occurred differently according to carbohydrate source and salivary condition. However, in each new and aged resin group, the comparison between 5 groups according to resin type can lead to an understanding of the tendency in their antibacterial property changes.

When glucose was used as carbohydrate source, regardless of the salivary condition, all experimental groups showed significantly higher antibacterial effects than the control in new resin group. On the contrary, there was no significant difference between the BAG group and the control in the aged resin group, with the exception of BAG + UA Monomer group which showed significantly higher antibacterial effects than the control group.

When sucrose was used as carbohydrate source, the group where UA was added to the monomer showed significantly high antibacterial effect in the new resin category, but in the aged resin category, there was no significant difference in all groups under saliva non-coating condition.

However, while there was no significant difference in the group where only BAG was included in the aged resin category, the group with UA added to the monomer showed

significantly higher antibacterial properties than the control group in the aged resin category. Therefore, we can see that the composite with UA added to the monomer maintains its antibacterial properties even after 6-month storage in distilled water.

This result is due to difference of the antibacterial mechanism between BAG and UA. BAG manifests its antibacterial properties by releasing alkaline and alkaline earth ions in an aqueous environment (Kozai et al., 1987). The experimental composite resin used in this study is BAG filler with fluoride. Previous study explained that antibacterial effect of BAG groups was due to the release of fluoride ions (Kim, 2013). To prove this, in this study, we evaluated whether fluoride is actually released in BAG-containing resin by ion chromatography. Actually, there was continuous fluoride ion release on new resin group, but it was not released on aged resin group. It can be assumed that all fluoride ions were released from composite disk surface after 6 month storage in distilled water.

BAGs do not containing fluoride manifests its antibacterial effect by release of several ions such as silicate, calcium, phosphorus, and sodium from the glass in an aqueous environment, resulting in an increase in pH (Stoor et al., 1998). It is speculated that anticariogenic ions in BAG had leached out these ions even after the long-term storage in distilled water.

On the contrary, the UA didn't leach out in the surrounding area. On previous study (Kim, 2013), the results of biofilm assay which is based on direct and close contact between the test microorganism and the surface of test materials was that UA had antibacterial effect. For this reason, UA must be in direct contact with the bacteria to manifest its antibacterial effects. The method of a number of study that UA showed antibacterial effect was almost biofilm assay (Kurek et al., 2012; Ren et al., 2005; Zhou et al., 2012).

In current study, the result of antibacterial effect on new resin group was different from previous study (Kim, 2013). Previous study showed that experimental BAG groups had

initial antibacterial effect against *S. mutans* biofilm formation comparing to control, and UA showed additional effects on reducing biofilm formation. Therefore, BAG + UA monomer group reduced the amount of biofilm formation significantly when glucose was given as a carbohydrate source. When sucrose was given, treatment with ursolic acid did not show any additional effects of decreasing biofilm formation. In this study, there were no significant differences between all experimental groups in new resin group under all conditions. The first probable cause is the difference in statistical method. In the previous study, a 2-way ANOVA with Bonferroni correction was applied. The second probable cause is the difference in experimental condition. In the previous study, 500 μL medium containing carbohydrate source and *S. mutans* suspension was used. However, the CFU value in this study was obtained by inoculating and incubating 1ml of medium, leading to CFU value which was relatively quite large compared to the previous study. Kozai et al(1987) showed that UA inhibited the insoluble glucan synthesis catalysed by a crude glucosyltransferase preparation from *S. mutans*. This antibacterial effect may be suppressed under 1mL medium.

There are several limitations in the present study. First, the changes in the antibacterial effect of composite disks were evaluated after storage in distilled water, but this is quite different from actual intraoral environment. When stored in artificial saliva, the changes in antibacterial effect of BAG by ion release and uptake may show different results. Second, after storage in distilled water, due to the speculation that the changes in biofilm inhibition effect is somewhat linked to surface energy. If we could measure the surface energy of the composite disk, it may be possible to interpret the result of sucrose under saliva coating condition. Third, 6 month is not enough time to evaluate the long-term effect of these composite resins, and further studies are needed.

V. Conclusion

In glucose source, experimental new composites containing BAG and/or UA showed significant reduction of biofilm formation by S. *mutans*. However, after storage in distilled water for 6 months, experimental composites containing BAG showed decreased biofilm inhibition effect. The composites with UA added to the monomer still showed significant inhibition effect of the biofilm formation by *S. mutans* even after storage in distilled water. In sucrose source, the new composites of UA Monomer group showed significant antibacterial effect under any salivary treatment. After storage in distilled water for 6 months, the biofilm formation was affected by salivary treatment. In Saliva non-coating groups, there were no significant difference in all groups, and in saliva coating groups, BAG + UA Monomer and UA BAG + UA Monomer groups showed lower CFU values.

Following the results of this experiment, it can be concluded that the UA incorporated in monomer was more effective method to keep the antibacterial effect in any biofilm formation condition after 6 month water storage condition.

Within the limitation of this experiment, this result indicates that UA inhibits biofilm formation by *S. mutans* and suggests that UA has potential for use as an effective antibacterial agent to prevent dental caries in the future.

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국 문 요 약

Bioactive glass 와 ursolic acid 를 함유한 복합 레진의 6개월 증류수 저장 후 *Streptococcus mutans* 바이오 필름에 대한 항균효과의 변화

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김 수 민

1. 목적

졸-겔 생활성(生活性) 유리와 트리테르페노이드를 다양한 방법으로 첨가한 항균 복합 레진을 증류수에 6 개월동안 노출시켰을 때 *Streptococcus mutans* 에 대한 항균 효과에 변화가 일어났는지 새로 제작한 항균 복합 레진과의 비교를 통해 알아보고자 하였다.

2. 재료 및 방법

충진재로 졸-겔 생활성 유리를 첨가한 복합레진 4 가지를 제작하였다. 생활성 유리군과 생활성 유리 충진재에 우르솔릭산을 첨가한 군, 레진 기질에 우르솔릭산을 첨가한 군, 그리고 생활성 유리 충진재와 레진 기질 양쪽 모두에 우르솔릭산을 첨가한 군을 실험 군으로 하여 대조 군의 일반 복합 레진과 비교하였다. 이전 실험 (Kim, 2013)에서 사용하였던 복합 레진 시편을 6 개월동안 증류수에 저장한 뒤, EO gas 소독을 시행하였다. 항균 작용을 평가하기 위하여 바이오필름 평가를 시행하였고 새로 제작한 레진 시편에 대해서도 동일한 조건하에서 바이오필름 평가를 시행하였다. 바이오필름 평가를 위해 시편을 여과한 비자극 타액 또는 PBS

(phosphate buffered saline) 로 코팅 후 포도당 또는 자당 배지를 사용하였다. 복합 레진 시편 상에서 *S. mutans* 를 24 시간동안 배양하였다. 세균 배양된 시편을 음파 처리하여 시편으로부터 세균을 분리한 후 집락 형성 단위를 측정하였다.

숙성된 항균 레진 시편과 새로 제작한 항균 레진 시편에서 방출되는 불소와 칼슘 이온의 농도를 정량적으로 측정하기 위해서 이온 크로마토그래피 평가를 시행하였다. 레진 종류에 따라 각각 5 개의 시편을 5mL 의 증류수에 24 시간동안 침전시킨 후 하루 동안 방출되는 이온 농도를 측정하였고, 농도 측정 후 새로운 증류수에 시편을 다시 침전시켰다. 이를 1 일 단위로 7 일까지 반복 측정하였다.

바이오필름 평가 후 새로 제작한 항균 레진 시편과 6 개월동안 숙성시킨 항균 레진 시편 사이의 항균 효과를 통계적으로 비교하기 위하여 t-test 를 사용하였다 ($p=0.05$). 또한 one-way ANOVA 및 다중 비교를 위한 Tukey test 를 사용하여 5 가지 레진 종류에 따른 항균 효과를 통계적으로 분석하였다 ($p=0.05$). 숙성된 항균 레진 시편과 새로 제작한 항균 레진 시편에서 방출되는 불소와 칼슘 이온의 농도를 통계적으로 비교하기 위하여 t- test 를 사용하였다 ($p=0.05$).

3. 결과

포도당 배지에서 타액 코팅 하지 않은 환경에서는 새 레진 시편과 숙성된 레진 시편간의 유의차는 보이지 않았다. 새 레진 시편에서는 모든 실험군이 대조군보다 유의차 있게 낮은 집락 형성 단위를 보였으나, 숙성된 레진 시편에서는 생활성 유리 충진재와 레진 기질에만 우르솔릭산을 첨가한 군 (BAG + UA Monomer)만 대조군보다 유의차 있게 낮은 집락 형성 단위를 보였다. 타액 코팅 하에서는 숙성된 레진 시편에서 모든 새 레진 시편보다 유의차 있게 높은 집락 형성 단위를 보였다. 새 레진 시편에서는 모든 실험군에서 대조군보다 유의차 있게 낮은 집락 형성 단위를 보였으나, 숙성된 레진 시편에서 생활성 유리군만 대조군과 집락 형성 단위에 유의차를 보이지 않았다.

자당 배지에서는 타액 코팅하지 않은 환경에서, 대조군을 제외한 모든 실험군에서 숙성된 레진 시편이 새 레진 시편보다 유의차 있게 높은 집락 형성 단위를 보였다. 새 레진 시편에서는 우르솔릭산을 레진 기질에 첨가한 군 (BAG + UA Monomer, UA BAG + UA Monomer)만 대조군보다 유의차 있게 낮은 집락 형성 단위를 보였으나, 숙성된 레진 시편에서는 모든 군간의 유의차는 보이지 않았다. 타액 코팅한 환경에서는 새 레진 시편과 숙성된 레진 시편 모두 우르솔릭산을 레진 기질에 첨가한 군 (BAG + UA Monomer, UA BAG + UA Monomer)만 대조군보다 유의차 있게 낮은 집락 형성 단위를 보였다.

레진 시편에서 방출된 이온의 농도를 측정하였을 때, 칼슘과 불소 이온 모두 새 레진 시편에서 숙성된 레진 시편보다 높은 농도의 이온이 방출되었다. 불소 이온은 새 레진 시편에서 모든 기간 동안 숙성된 레진 시편보다 유의차 있게 높은 양의 이온이 방출되었다. 칼슘이온은 1, 5 일째에서만 새 레진 시편이 숙성된 레진 시편보다 유의차 있게 높은 이온이 방출되었다. 칼슘과 불소 이온 모두 새 레진 시편에서 하루 동안 방출된 이온의 농도는 1 일 째 가장 높았고, 2 일 째 감소하였다가 3 일 째 다시 증가하였다.

4. 결론

포도당 배지 하에서, 생활성 유리와 우르솔릭산을 함유한 항균 레진은 새 레진 시편에서 *S. mutans* 의 바이오필름 형성을 유의차 있게 감소시켰다. 그러나 6 개월동안 증류수에 저장 후, 생활성 유리를 포함한 항균 레진의 바이오필름 억제 능력이 감소함을 보였다. 우르솔릭산을 레진 기질에 포함한 항균 레진은 증류수에 저장 후에도 *S. mutans* 바이오필름에 대한 항균 효과가 지속됨을 보였다.

자당 배지에서는 우르솔릭산을 레진 기질에 첨가한 항균 레진은 새 레진 시편에서 타액 코팅 여부에 관계 없이 대조군에 비해 유의차 있게 항균효과가 있음을 보였다. 6 개월 증류수에 저장 후, 타액 코팅하지 않은 환경에서는 모든 군간 항균 효과에 유의차는 발견되지 않았다. 타액 코팅

환경에서는 우르솔릭산을 레진 기질에 첨가한 항균 레진에서 바이오필름에 대한 항균 효과가 지속됨을 보였다.

이로써 우르솔릭산을 레진 기질에 포함한 군은 6 개월동안 증류수에 저장한 이후에도 항균성을 유지하는 경향을 보임을 결론지을 수 있다. 생활성 유리군은 증류수에 저장 하는 동안 항균성을 가지는 이온의 방출로 인하여 장기간의 항균 효과가 감소함을 알 수 있었다.

실험의 한계에도 불구하고, 이러한 결과를 통하여 우르솔릭산은 *S.mutans* 의 바이오필름 형성을 장기적으로 억제하므로 치아 우식을 예방하기 위한 항균 제재로 효과적으로 사용할 수 있을 것이다.

Key words : 생활성 유리, 우르솔릭산, 항균 복합레진, 바이오필름,
Streptococcus mutans, 숙성된 항균 복합레진, 증류수 저장