Effects of cigarette smoking on the growth of *Streptococcus mutans* biofilms: an in vitro study

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Abstract

This study aimed to investigate the differences in growth and virulence (EPSs and acidogenicity) of *Streptococcus mutans* biofilms (*S. mutans*) according to the different times of cigarette smoking (CS) treatment. *S. mutans* biofilms (74-hour-old) were formed on saliva-coated hydroxyapatite disks. The biofilms were treated with CS at different times per day (one time, three times, and six times/day). The control group did not receive CS treatment. Acidogenicity, dry weight, colony-forming units, water-soluble/insoluble extracellular polysaccharides, and intracellular polysaccharides were analyzed and confocal laser scanning microscopy images were obtained of the 74-h-old biofilms. The 74-h-old biofilms on sHA discs in the 6 times/day CS treatment group showed the lowest biofilm accumulation and extracellular polysaccharide amount compared with the control group and other CS

treatment groups. In the CLSM study, the biofilms in the six times/day CS treatment group also showed the lowest bacterial count (live and dead cells) and EPS biovolume. CS has an obvious inhibition on the growth of *S. mutans* biofilms, the degree of inhibition is proportional to the number of CS treatments.

Introduction

Dental caries is a non-infectious multivariate oral disease associated with dental biofilm. An important contributing factor in the development of dental caries is the change of microbial properties in dental biofilm. The increase of aciduric and acidogenic bacteria in the microorganism plays an important role in the pathogenesis of dental caries[1]. When biofilms exist on the surface of teeth and are exposed to dietary sugar, acidogenic bacteria reduce pH in the dental biofilm by means of glycolysis[1]. The low pH environment further increased the growth of acidity and acidogenic bacteria such as *S. mutans*[2]. Although the composition of cariogenic biofilm microflora is complex[3], *S. mutans* is the main pathogen of caries. *S. mutans* can synthesize extracellular polysaccharides (EPSs) and intracellular polysaccharides (IPSs) using sucrose as substrate. EPSs promotes bacterial adhesion on the tooth surface, colonization, and formation of plaque biofilm, which is an important cariogenic factor of *S. mutans*. The other major cariogenicity of *S. mutans* is determined by its acid production (using carbohydrates to produce acid) and acid tolerance (living at low pH), resulting in the loss of local hard tissue and the start of the cariogenic process[4].

Cigarette smoking (CS) is an important factor affecting the progress of dental caries. Numerous epidemiological studies around the world have reported a close relationship between smoking and the occurrence of dental caries[5, 6, 7, 8, 9]. An animal study reported that CS exposure expands the caries-affected area in the maxillary molars of the rat[10]. This is further confirmed by evidence from epidemiological studies associating CS with high caries prevalence[11]. CS is one factor with a potential impact on oral cavity microbial ecosystems[12].

To the best of the author's knowledge, there are few studies on the effects of CS on the growth of oral cariogenic biofilm (*S. mutans* biofilms). It is very important to study the influence of CS on the growth of

S. mutans biofilms and dynamic bacterial equilibrium for the development and prevention of dental caries in smokers. This study aimed to investigate the differences in growth, virulence (EPSs and acidogenicity), and viability of cariogenic biofilms according to the different times of CS (simulate different smoking times each day) using an *S. mutans* biofilm model.

Result

The difference inS. mutans**biofilms formation.** 74-h-old *S. mutans* biofilms formation was inhibited by CS treatment. In the experimental results of microbial and biochemical studies, all CS treatment groups showed inhibitory effects on the *S. mutans* biofilms formation. The dry weight (all biofilm components except water), water-insoluble extracellular polysaccharides (ASP), water-soluble extracellular polysaccharides (WSP), and intracellular polysaccharides (IPS) amount of 74-h-old *S. mutans* biofilms showed the lowest values in the six times/day CS treatment group compared with the control group and other CS treatment groups (Figs. 1A, 1C, 1D, 1E) (Figs. 1A, 1C, 1D, 1E, $p < 0.05$). There was no significant difference in CFUs count among all CS treatment groups (Fig. 1B) ($P > 0.05$).

The difference in acid production. Acid production in the 74-h-old *S. mutans* biofilms was also influenced by the CS treatment. The initial rate (0–20, 30 min) and the total rate of H^+ production (0–120 min) of 74-h-old *S. mutans* biofilms showed the lowest values in the six times/day CS treatment group compared with the control group and other CS treatment groups (Fig. 2) ($p > 0.05$).

The pH value in the culture medium. Figure 3 shows the change in pH of the culture medium by different times of CS treatments during 74-h-old *S. mutans* biofilms formation. Acidogenicity during mature *S. mutans* biofilms formation was affected by CS treatments. Although the acidogenicity of *S. mutans* biofilms before the CS treatments were not different from each other ($P > 0.05$) (0-21h of biofilm formation). But the pH in the culture medium started to change after treatment with CS, the acidogenicity was inhibited at all of the CS tested groups ($P < 0.05$) (21-74h). The pH value of the culture medium was increased as CS treatment times increased. The pH value in the culture medium of six times/day CS treatment group was higher than those compared with other CS treatment groups and control group ($P < 0.05$). Furthermore, in all of the CS treatment periods and non-CS treatment periods (21-74h), the pH value of the culture medium of *S. mutans* biofilms in 6 times/day CS treatment group was significantly different from the control group ($P < 0.05$).

Bacterial biovolume and thickness. To further evaluate the effect of CS on biofilm components and structure, the CLSM analysis was performed. The bacterial biovolume and thickness of live or dead cells of the 74-h-old *S. mutans* biofilms showed different results under different times of CS treatment (Fig. 4). The bacterial biovolume and thickness of live or dead cells and total biovolume of the 74-h-old *S. mutans* biofilms in the 6 times/day CS treatment group showed the lowest values compared with the other CS treatment groups and control group (Figs. 4A, 4B, 4D, $p < 0.05$). In the percent of total biovolume, the proportion of live cell biovolume in each treatment group was between 60% and 70% (Fig. 4E). In each treatment group, the biovolume and thickness of live cells were higher than those of dead cells.

Representative three-dimensional images of live or dead cells were showed in Fig. 4C, the 74-h-old *S. mutans* biofilms in the six times/day CS treatment group showed the lowest biofilm volume compared with the other CS treatment groups and control group (Fig. 4C).

EPS biovolume and thickness. The biovolume and thickness of EPSs in the 74-h-old *S. mutans* biofilms were also influenced by the different times of CS treatment. 74-h-old *S. mutans* biofilms formation was strongly influenced by the six times/day CS treatment. The mean biovolume and thickness of EPSs of the 74-h-old *S. mutans* biofilms in the six times/day CS treatment group showed the lowest values compared with the other CS treatment groups and control group (Figs. 5B, C) ($p < 0.05$). The representative three-dimensional images of EPSs showed that the six times/day treatment group exhibited the lowest concentrations of EPSs compared with the other CS treatment groups and control group (Fig. 5A).

The difference in biofilm density. The density of *S. mutan*s biofilms was calculated to investigate the difference in biofilm compactness according to different times of CS treatment. As shown in Fig. 6, there was no significant difference in biofilm density in all the CS treatment groups and the control group ($p > 0.05$).

Figure 7 showed the surface morphology of each group of hydroxyapatite disk after 74-h-old incubation. The surface of the hydroxyapatite disk in the six times/day CS treatment group showed the largest area of the yellow film (a mixture of chemical substances produced by cigarette combustion) covering compared to the other CS treatment groups and the control group. And the accumulation of biofilm on the surface of the yellow film covering the area was less than that on the surface of white hydroxyapatite.

Discussion

This study was to investigate the differences in growth, virulence (EPSs and acidogenicity), and viability of cariogenic biofilms according to the different times of CS treatment (simulate different smoking times each day) using an *S. mutans* biofilm model. This experiment appears to be the first to study the relationship between CS and the growth of *S. mutans* biofilms using an in vitro device that simulates the process of oral smoking.

The major outcome from this study was CS inhibited the growth of *S. mutans* biofilms in vitro, which was well supported by measurements of biofilm dry weight (Fig. 1A), EPS (Fig. 1C, 1D; Fig. 5), IPS (Fig. 1E), acidogenic (Fig. 2; Fig. 3), bacterial count (live or dead cells) (Fig. 4A) and total biovolume (Fig. 4D). The inhibition degree of CS on biofilm growth was directly proportional to the number of CS treatments. The six times/day CS treatment group showed the highest inhibition compared to the control group, one time/day CS treatment group, and three times/day CS treatment group.

In the results of CFU counts (Fig. 1B) and biofilm density (Fig. 6), there was no significant difference between all CS treatment groups and the control group. Meanwhile, in the results of CLSM, the ratio of live cells and dead cells in the total biovolume (Fig. 4E) was similar between all CS treatment groups and

the control group. These results suggested that CS does not inhibit the growth of *S. mutans* biofilms by sterilizing or killing cells.

The hydroxyapatite disks in all CS treatment groups after 74-h-old incubation showed that the surface of the hydroxyapatite disks covered with a yellow film (Fig. 7), which formed when a mixture of chemical substances produced by cigarette combustion attached to the surface of the hydroxyapatite disks. Cigarette combustion produces more than five thousand chemicals and contains a variety of harmful substances[13,14,15,16]. In the six times/day treatment group, the area of the yellow film on the surface of the hydroxyapatite disk was significantly larger than that of other CS treatment groups and control groups (Fig. 7). Compared with the normal white hydroxyapatite disk in the control group, the accumulation of biofilms on the yellow film surface was lower than that on the normal hydroxyapatite disk surface, and even the formation of *S. mutans* biofilms could not be seen on some area of the yellow film surface. This may be due to the fact that a mixture of chemical substances produced by cigarette combustion attached to the surface of the hydroxyapatite disc, changing the nature of the original hydroxyapatite disc and *S. mutans* biofilms are difficult to colonize on the surface of the chemical mixture. In the experiment, with the increase of CS treatment times, the new mixture of chemical substances produced by cigarette combustion continuously covered the surface of *S. mutans* biofilms, inhibited the growth of *S. mutans* biofilms, and inhibited the new colonization of *S. mutans* biofilm on the surface of the chemical mixture.

In the hydroxyapatite disks of the CS treatment groups, with the increase of the number of CS treatments, the available area for the colonization and growth of *S. mutans* biofilms became smaller and smaller. So in the six times/day CS treatment group showed the lowest biofilm dry weight, EPS, IPS, acidogenic bacterial count (live or dead cells), and total biovolume, while the areas not covered by the chemical mixture showed similar CFU counts, the ratio of live cells and dead cells in the total biovolume and biofilm density as the control group. In the CLSM results, the thickness of bacteria (live or dead cells) was inversely proportional to the increase in the number of CS treatments, and the lowest bacterial thickness was shown in the six times/day CS treatment group, which also confirmed that the mixture of chemical substances produced by cigarette combustion continuously covered on the surface of the *S. mutans* biofilms, which inhibited the increase of bacterial thickness (Fig. 4B). The mixture of chemical substances produced by cigarette combustion is attached to the surface of the hydroxyapatite disk, which also changes the color of the disk. In practice, when smoking in the mouth, this mixture of chemical substances attached to the surface of the teeth and caused a change in the color of the smoker's teeth[17].

In general, the results in this study supported that CS inhibited the growth of *S. mutans* biofilms, the mixture of chemical substances produced by cigarette combustion changed the colonizing environment on the surface of the hydroxyapatite disc, inhibited the growth of *S. mutans* biofilms. These results indicated that the mechanism of action between CS and high caries incidence was not to promote the cariogenic biofilm growth.

The main purpose of this experiment was to study the relationship between CS dose and the growth of oral cariogenic bacteria. In vitro experiments avoided the interference of other influencing factors as much as possible. The primary variable was different doses of CS, and a representative oral cariogenic bacteria (*S. mutans*) was selected. Although *S. mutans* is an important cariogenic bacteria, considering the complex relationship among oral bacteria, different bacteria may influence each other, and a single bacterial species can not represent all oral cariogenic bacteria. The effects of different kinds of cigarettes on the growth of oral cariogenic bacteria also need to be verified. Future research may aim to observe the interactions between more oral bacteria strains and different types of CS, especially among multiple bacteria strains treated with CS.

Conclusion

The CS significantly inhibited the growth of *S. mutans* biofilms. In the results of this experiment of microbiological and biochemical and CLSM, six times/day CS treatment group showed the highest inhibitory effect on *S. mutans* biofilms, even in the one time/day CS treatment group also showed inhibition.

Materials And Methods

***Streptococcus mutans* biofilms formation and cigarette smoking experimental scheme.** *Streptococcus mutans* UA159 (ATCC 700610; serotype c) biofilms were formed on saliva-coated hydroxyapatite (sHA) discs (2.93 cm^2; Clarkson Chromatography Products, Inc., South Williamsport, PA, USA) placed in a vertical position in 24-well plates. Briefly, an adult male (non-smoker) was selected for oral saliva collection. HA discs were incubated in filter-sterilized (0.22-μm low protein-binding filter) saliva (3 ml/disc) for 1 h at 37°C. For biofilms formation, the sHA discs were transferred to a 24-well plate containing brain heart infusion (BHI; D-ifco, Detroit, MI, USA) broth with 1% (w/v) sucrose and *S. mutans* UA159 ($5–7×10^6$ colony-forming unit (CFU)/ml) (3 ml/disc). The biofilms were grown at 37°C with 5% CO_2 for 21 h to allow initial biofilms growth. After 21 h, *S. mutans* biofilms were divided into 4 groups. Experiment group 1 (control group) did not receive CS treatment and exposed to the air six times per day (at 8, 10, 12 a.m., 2, 4, 6 p.m.). Experiment group 2 was treated one time per day (at 10 a.m.) with CS and five times per day (at 8, 12 a.m., 2, 4, 6 p.m.) with air, a total of three times CS treatments in 74-h-old biofilms. Experiment group 3 was treated three times per day (at 10 a.m., 2, 6 p.m.) with CS and three times per day (at 8, 12 a.m., 4 p.m.) with air, a total of seven times CS treatments in 74-h-old biofilms. Experiment group 4 was treated six times per day (at 8, 10, 12 a.m., 2, 4, 6 p.m.) with CS, a total of fifteen times treatments in 74-h-old biofilms. Each treatment time was 5 minutes (simulate the real smoking time of smokers). The culture medium was changed twice daily (8 a.m. and 6 p.m.) (Oral sugar levels rise after 8 a.m. for breakfast and 6 p.m. for dinner). The hydroxyapatite disks were washed with distilled water three times a day (8 a.m., 1 p.m., 6 p.m.) (Simulate cleaning mouth after breakfast, lunch, and dinner) for the control group and all CS treatment groups. The incubated time of the *S. mutans* biofilms was 74 hours (Fig. 8). This study is approved by the ethics committee/institutional review board of the

Department of Preventive Dentistry, School of Dentistry, Institute of Oral Bioscience, Jeonbuk National University. All experimental protocols were approved by the Department of Preventive Dentistry, School of Dentistry, Institute of Oral Bioscience, Jeonbuk National University. The author confirms that all methods were carried out in accordance with relevant guidelines and regulations. The author confirms that informed consent had been obtained from all subjects.

The microenvironment of plaque is easily affected by various bacteria and external factors. To minimize the influence of external factors and improve the internal validity of the results, an in vitro method was considered. Considering that *S. mutans* is a facultative anaerobe, a glass container was designed to allow air to enter while CS was inhaled to avoid the effect of complete hypoxia on the growth of *S. mutans* biofilm. At the time of treatment, the sHA discs were taken out from the culture medium, placed in a sterile glass container, and a cigarette was taken using a vacuum machine to simulate cigarette gas in the mouth during smoking, each treatment time was 5 minutes. After treatment, sHA discs were returned to the culture medium (Fig. 9).

In this experiment, a popular cigarette in Korean supermarkets was selected. Marlboro (tar: 8.0 mg; nicotine: 0.7 mg), it has the highest tar and nicotine content per cigarette of all cigarette brand. We used vacuum machines to provide smoking force.

Microbiological and biochemical biofilm analyses. The dry weight and colony-forming units (CFUs) in the homogenized suspension were analyzed. Briefly, the 74-h-old biofilms on the sHA disc were transferred into 2 ml of 0.89% NaCl and sonicated in an ultrasonic bath for 10 min to disperse the biofilms. The dispersed solution was re-sonicated at 7W for 30 s after adding 3 ml of 0.89% NaCl (VCX 130PB; Sonics and Materials, Inc., Newtown, CT, USA). For the determination of CFUs count, an aliquot (0.1 ml) of the homogenized solution (5 ml) was serially diluted, plated onto brain heart infusion (BHI; Difco, Detroit, MI, USA) agar plates, and then incubated under aerobic conditions at 37°C to determine the CFUs count[18, 19].

For the determination of the dry weight and amount of water-insoluble extracellular polysaccharides (water-insoluble EPSs) (ASP), water-soluble extracellular polysaccharides (water-soluble EPSs) (WSP), intracellular polysaccharides (IPS), the remaining solution (4.9 ml) was centrifuged (3000 ×g) for 20 min at 4°C. The biofilm pellet was resuspended and washed twice in the same volume of water. Mix the water washed the biofilms pellet with 95% alcohol and put it in a refrigerator at -20°C for at least 18 hours to precipitate the water-soluble EPS. Then calculate the content of water-soluble EPS in the biofilms. The washed biofilms pellet was evenly divided into two portions, lyophilized, and weighed to determine the dry weight. One part used 1 N sodium hydroxide to extract water-insoluble EPS from the dried precipitate. The other part was used to calculate the content of intracellular polysaccharides, as detailed elsewhere[20].

The final pH values of the old culture media were also determined during the experimental period using a glass electrode (Beckman Coulter Inc., Brea, CA, USA) to investigate the change in acidogenicity of *S. mutans* biofilms by the treatments.

Glycolytic pH drop assay. To evaluate the activity of CS against acid production of *S. mutans* biofilms, a glycolytic pH drop assay was performed. Briefly, the 74-h-old *S. mutans* biofilms, which had been incubated in 20 mM potassium phosphate buffer (pH 7.2) for 1 h to deplete endogenous catabolites. And then transferred to a salt solution (50 mM KCl plus 1 mM $MgCl_2$, pH 7.0). The pH was adjusted to 7.2 with a 0.2 M KOH solution. Glucose was then added to the mixture to give a final concentration of 1% (w/v). The decrease in pH was assessed using a glass electrode over 120 min (Futura Micro Combination pH electrode, 5 mm diameter; Beckman Coulter Inc., CA, USA). The effect of CS on the acid production of the biofilm was determined according to the acid production rate, calculated by the change in pH values over the linear portion (0–20, 30, 120 min) of the pH drop curves[21].

Confocal laser scanning microscopy analysis

Live and dead bacterial cells staining. Confocal laser scanning microscopy (CLSM) analysis was performed to confirm the results of microbiological and biochemical studies. To investigate the difference in bacterial cells, the 74-h-treated biofilms were stained at room temperature in the dark for 30 min using the Film Tracer LIVE/DEAD Biofilm viability kit L10316 (Invitrogen, Molecular Probes Inc., Eugene, OR, USA). The final concentrations of SYTO®9 and propidium iodide (PI) were 6.0 and 30 μM, respectively. This viability kit was based on plasma membrane integrity to determine live and dead cells. In this study, we regarded the cells with intact membranes (green) as live cells, whereas cells with damaged membranes (red) were regarded as the dead cells. The excitation/emission wavelengths were 480/500nm for SYTO®9 and 490/635nm for PI for collecting the fluorescence. The stained live and dead bacterial cells were observed with an LSM 510 META microscope (Carl Zeiss, Jena, Germany) equipped with argon-ion and helium–neon lasers. All confocal fluorescence images were taken with an EC Plan-Neofuar 10x/0.30 M27 objective lens. A stack of slices in 6.4 μm step sizes was captured from the top to the bottom of the biofilms. The biovolume and thickness of live and dead cells were quantified from the entire stack using COMSTAT image-processing software. The biovolume is defined as the volume of the biomass (μm^3) divided by the substratum (hydroxyapatite surface) area (μm^2). The three-dimensional architecture of the biofilms was visualized using ZEN 2.3 (blue edition) (Carl Zeiss Microscopy GmbH, Jena, Germany). The original confocal data was uploaded to ZEN 2.3 software and the intensity of green and red fluorescence in the full thickness of biofilms layers were captured automatically. The software reconstructed the 2-dimensional intensity of fluorescence in all the layers to a 3-dimensional volume stack[22].

EPS staining. The EPSs of 74-h-old biofilms were also investigated by simultaneous in situ labeling as described elsewhere[23]. Briefly, Alexa Fluor® 647-labeled dextran conjugate (1 μM, 10,000 MW; absorbance/fluorescence emission maxima 647/668 nm; Molecular Probes Inc., Eugene, OR, USA) was added to the culture medium during the formation of *S. mutans* biofilms (at 0, 21, 31, 45, 55, 69 h) to label the newly formed EPSs. As described above, the stained EPSs were observed with an LSM 510 META microscope (Carl Zeiss, Jena, Germany) (objective: EC Plan Neofuar 10x/0.30 M27) equipped with argon-ion and helium-neon lasers and visualized using ZEN 2.3. A stack of slices in 7.8 μm step sizes was

captured from the top to the bottom of the biofilms. Four independent experiments were performed, and five image stacks per experiment were collected. The EPSs biovolume and thickness were quantified from the confocal stacks using COMSTAT.

Biofilm density. The density of the 74-h-old *S. mutans* biofilms was calculated using the dry weight, which was derived from the biochemical study above, and the total biovolume of the biofilms (live cells + dead cells + EPSs), which was derived from the CLSM study above. The biofilm density ($\mu g/\mu m^3$) is defined as the dry weight ($\mu g/\mu m^2$) divided by the total biovolume of the biofilms ($\mu m^3/\mu m^2$)[24].

Statistical Analysis. All experiments (except CLSM and SEM) were performed in duplicate, and at least six different experiments were conducted. The data are presented as mean ± standard deviation. Inter-group differences were estimated using one-way analysis of variance, followed by a post hoc multiple comparison (Tukey) test to compare multiple means (SPSS® software, IBM). Values were considered statistically significant when the p-value was < 0.05.

Declarations

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Author Contributions:

Ye Han designed this study, performed the experiments, analyzed the data, and wrote the main manuscript text.

Author shave reviewed and approved the complete manuscript for submission.

Additional Information

Conflict of Interest Statement

The author has no conflicts of interest to declare.

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Figures

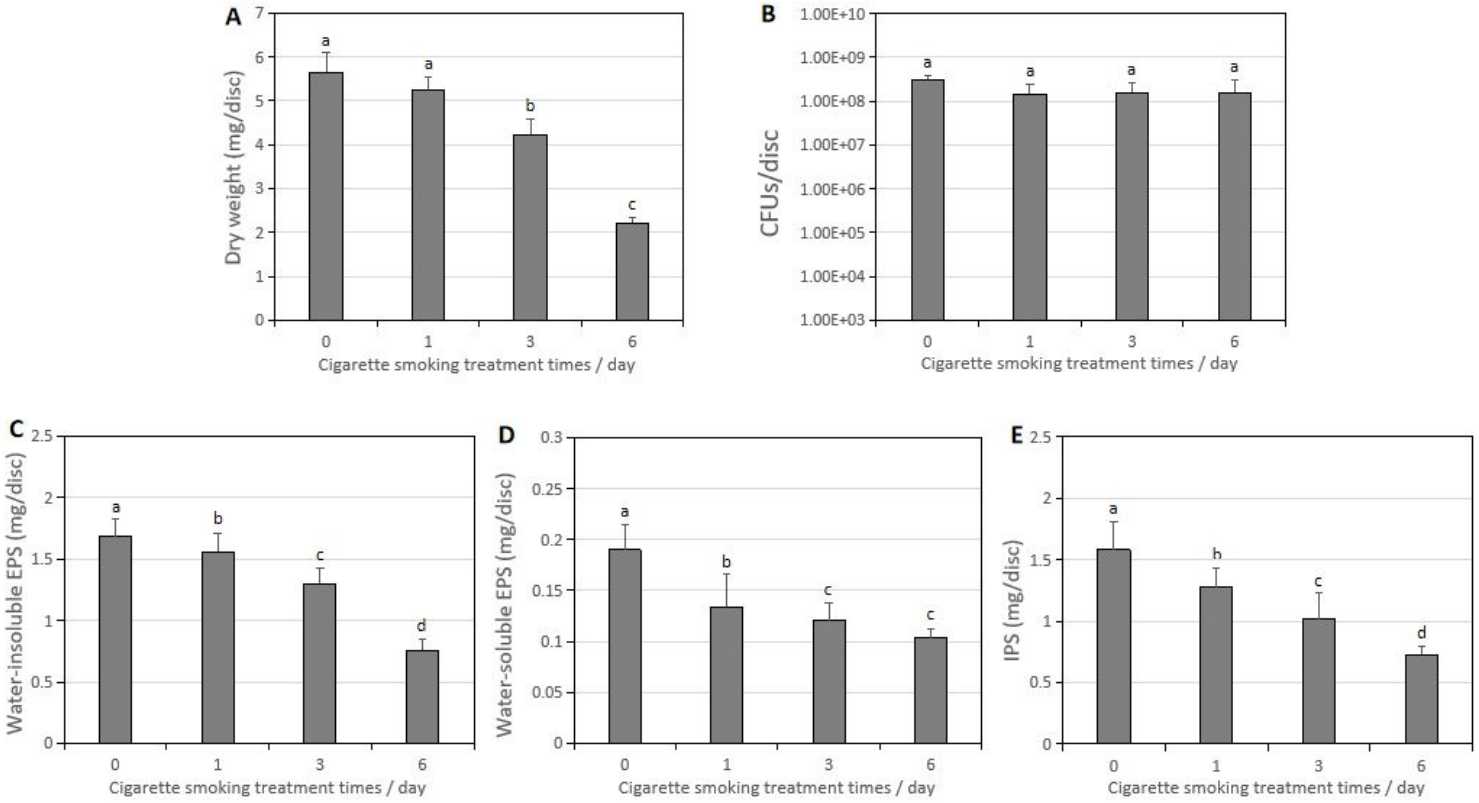
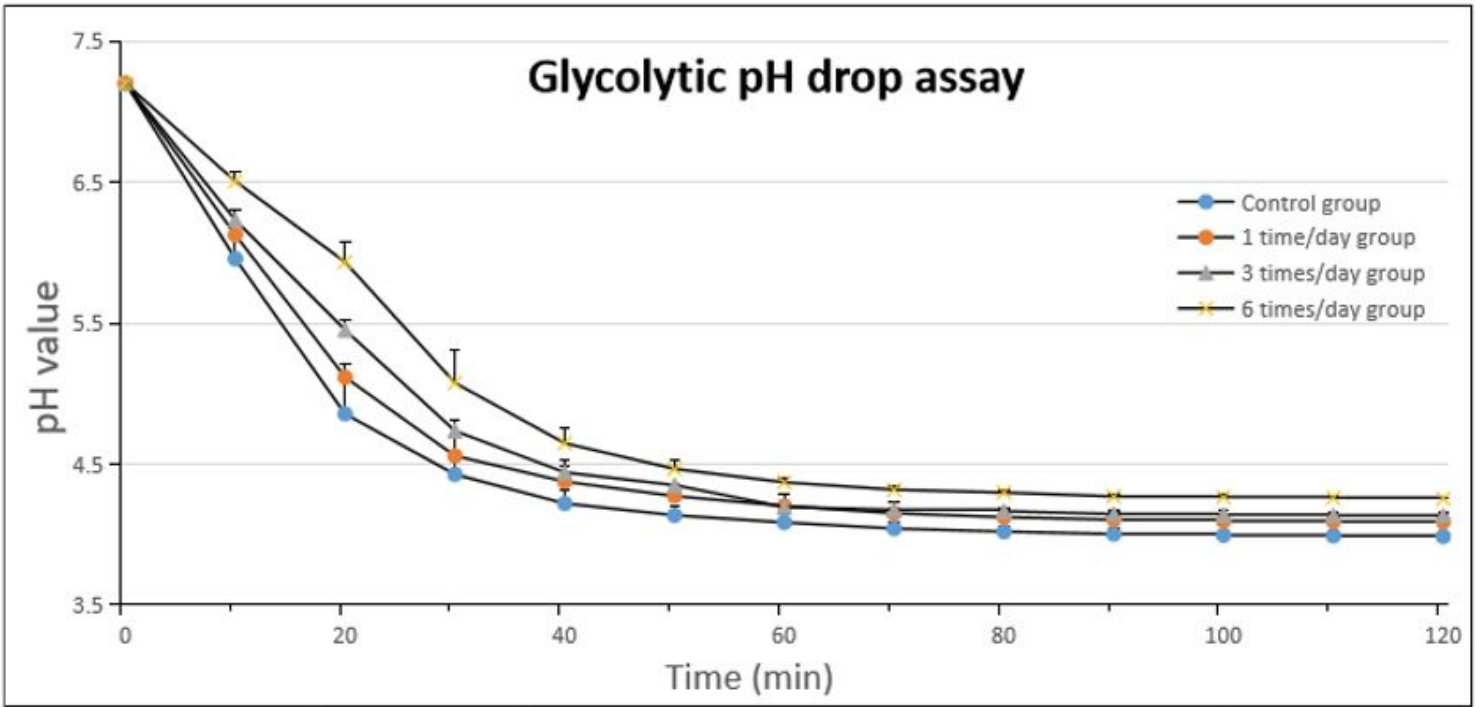


Figure 1

Change in the microbiological and biochemical composition of the 74-h-old S. mutans UA159 biofilms treated with cigarette smoking. (A) Dry weight. (B) CFUs. (C) Water-insoluble EPS. (D) Water-soluble EPS. (E) Intracellular polysaccharides (IPS). Data represent mean ± standard deviation. Figure 1A, 1C, 1D, 1E, $p < 0.05$. Figure 1B, $p > 0.05$ *$P < 0.05$: significantly different from each other. $P > 0.05$: value followed by the same superscript are not significantly different from each other.



Initial rate of pH drop (amount of pH drop/min)				
	Control group	1 time/day group	3 times/day group	6 times/day group
0-20min	0.1172	0.1042	0.0875	0.0634
0-30min	0.0924	0.0880	0.0821	0.0708
Total rate of pH drop (amount of pH drop/min)				
	Control group	1 time/day group	3 times/day group	6 times/day group
0-120 min	0.02678	0.02592	0.02554	0.02456

Figure 2

The effect of cigarette smoking treatment on the glycolytic pH drop of 74-hour-old S. mutans UA159 biofilms cells. Data represent mean ± standard deviation. Values followed by the same superscript are not significantly different from each other ($p > 0.05$).

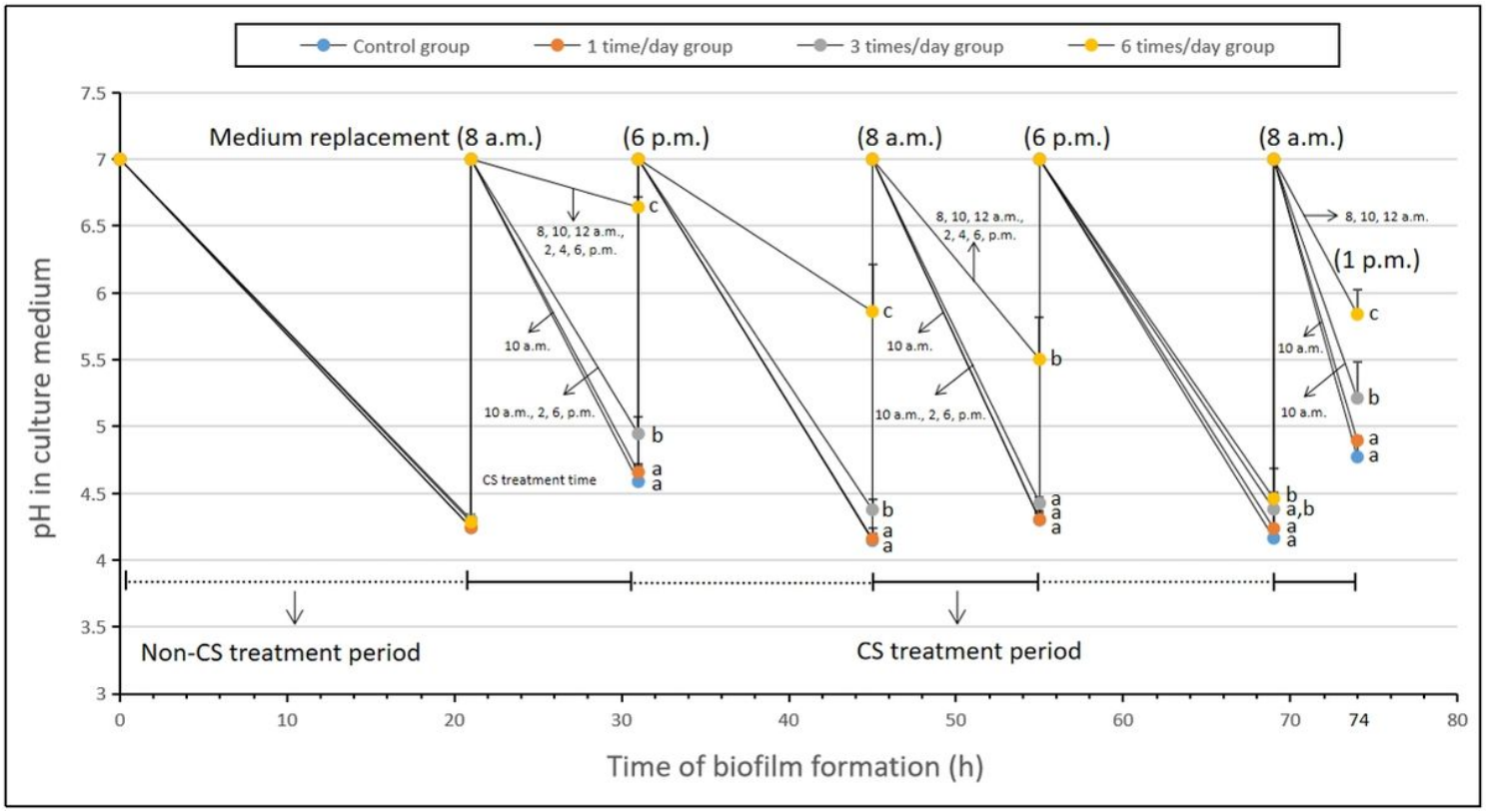


Figure 3

Change in the pH values of old culture medium treated with cigarette smoking during 74-hour S. mutans UA159 biofilms formation. Data represent mean ± standard deviation. Values followed by the same superscript are not significantly different from each other ($p > 0.05$).

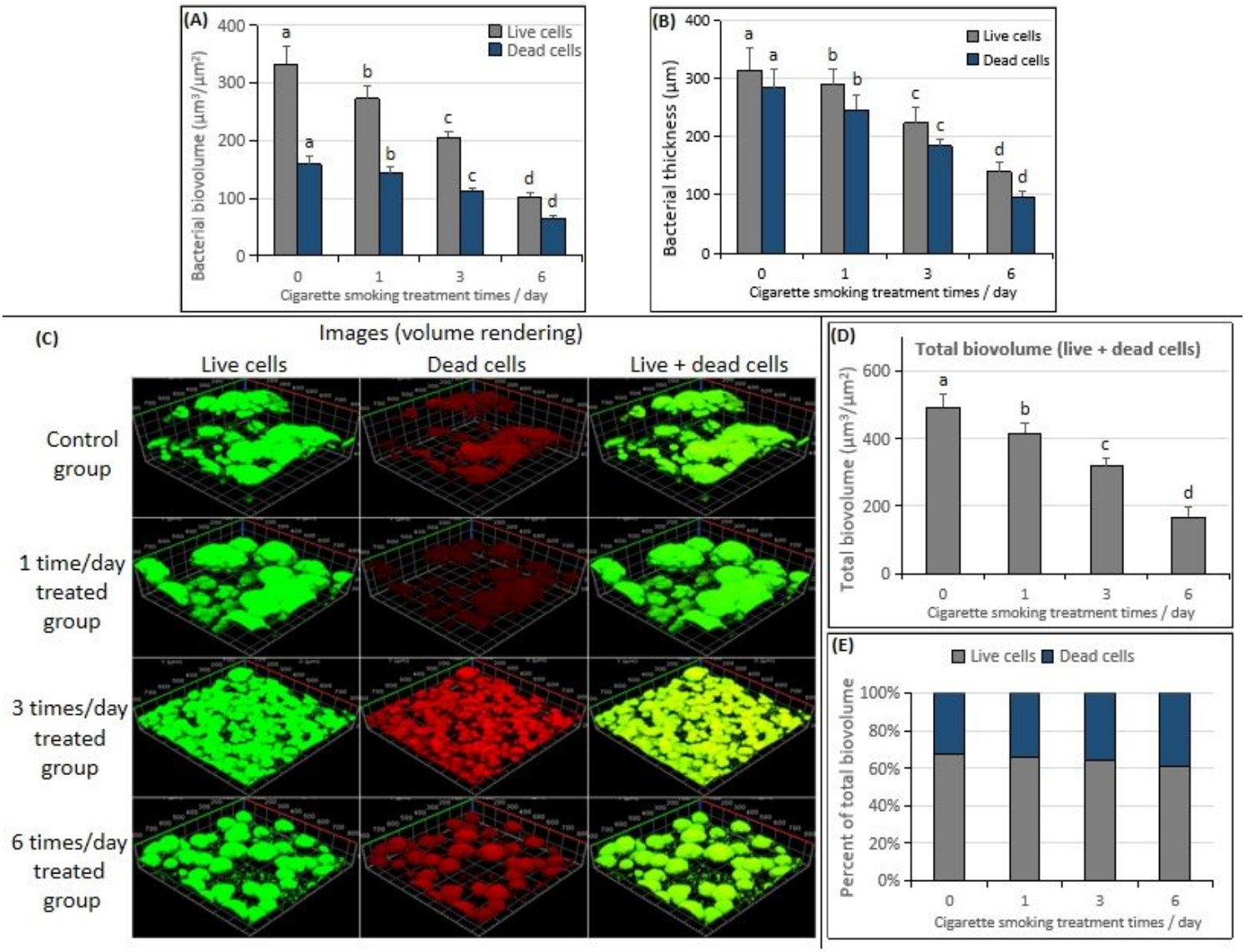


Figure 4

Change in CLSM of bacterial cells in the 74-h-old S. mutans UA159 biofilms treated with cigarette smoking. (A) Bacterial biovolume. (B) Bacterial thickness. (C) Representative confocal images. (D) Total biovolume (live + dead cells). (E) Percent of total bio-volume. Significantly different from each other ($p < 0.05$).

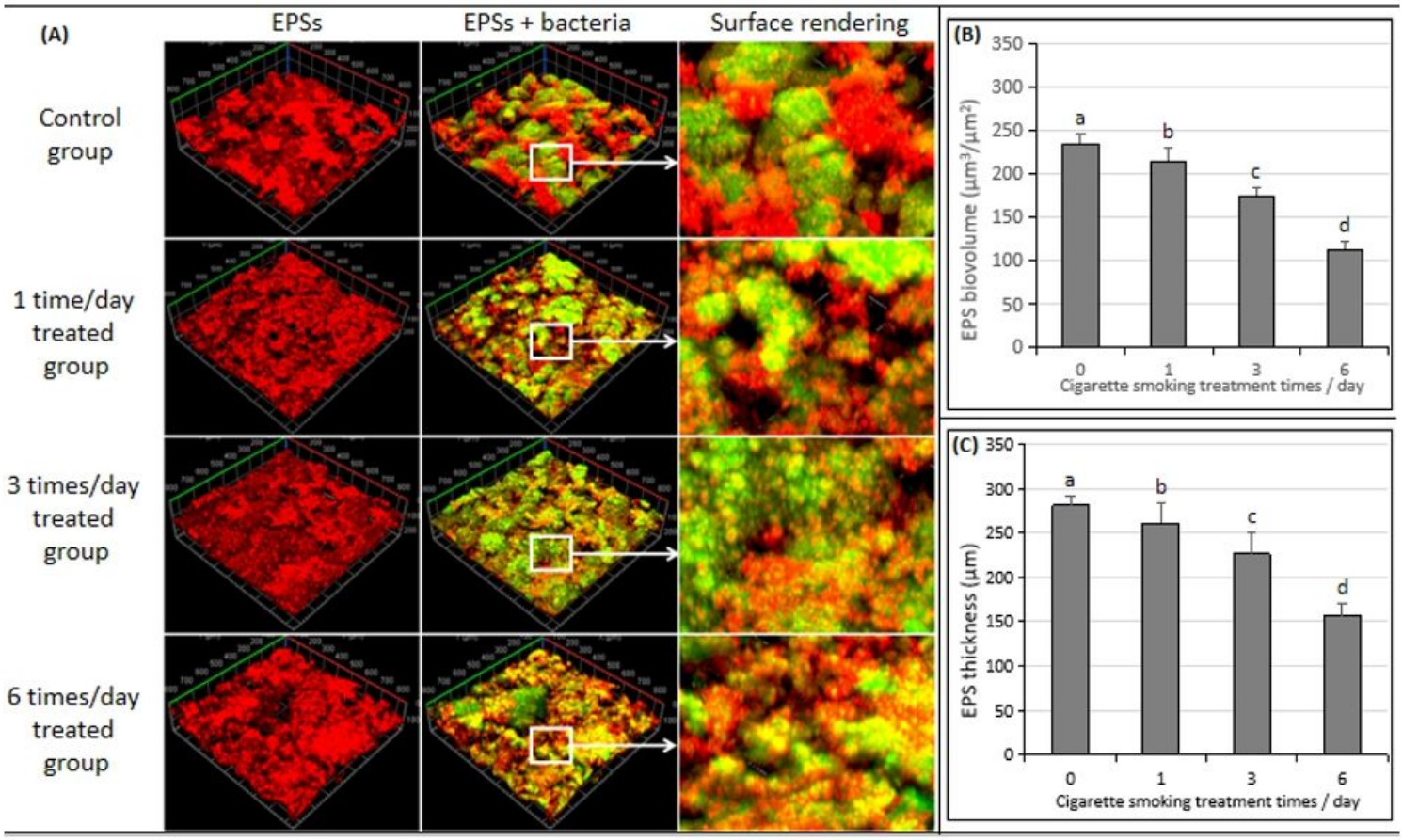


Figure 5

Change in CLSM of EPSs in the 74-h-old S. mutans UA159 biofilms treated with cigarette smoking. (A) EPS biovolume. (B) EPS thickness. (C) Representative confocal images. Significantly different from each other ($p < 0.05$).

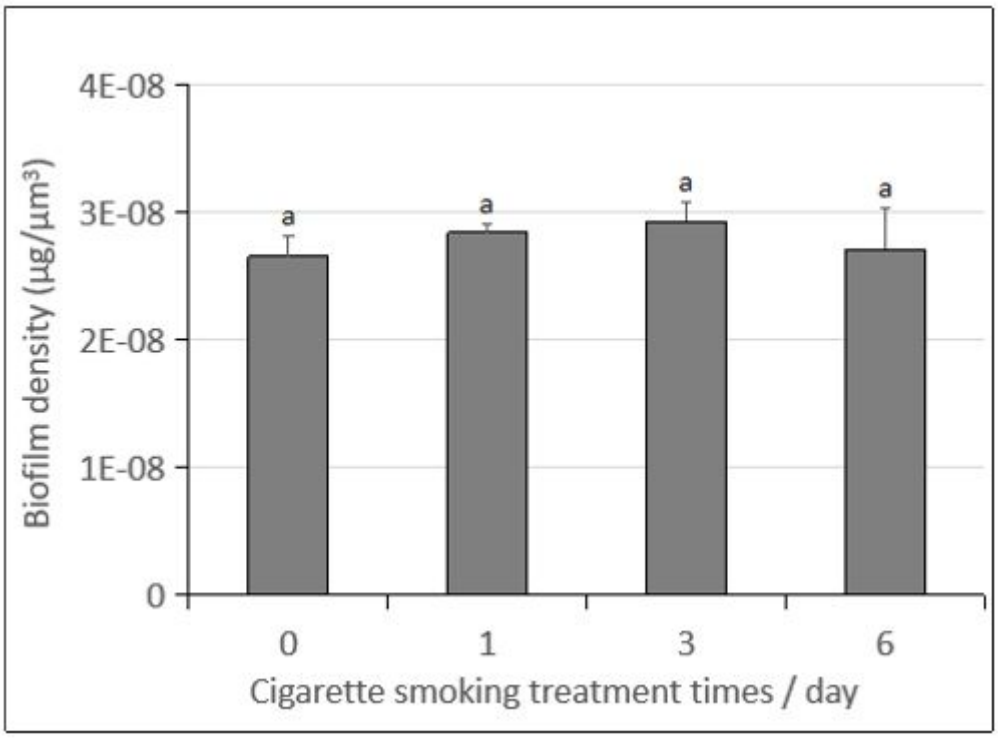


Figure 6

Change in the bioflm density of 74-h-old S. mutans UA159 biofilms treated with cigarette smoking. Values followed by the same superscripts are not significantly different from each other ($p > 0.05$).

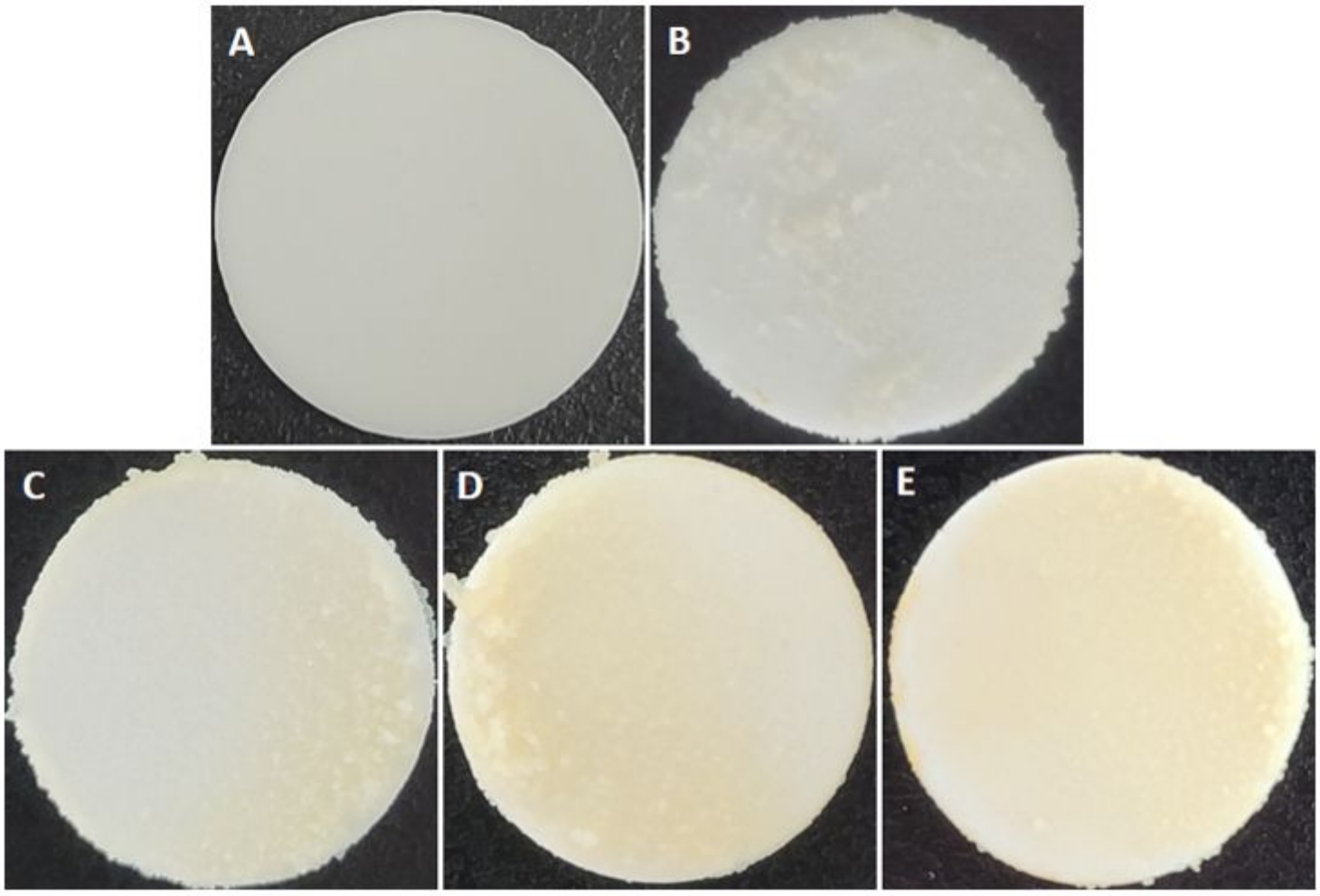


Figure 7

Surface morphology of hydroxyapatite disks before the experiment and each group after 74-h-old incubation. (A) Before the experiment. (B) Control group. (C) 1 time/day CS treatment group. (D) 3 times/day CS treatment group. (E) 6 times/day CS treatment group.

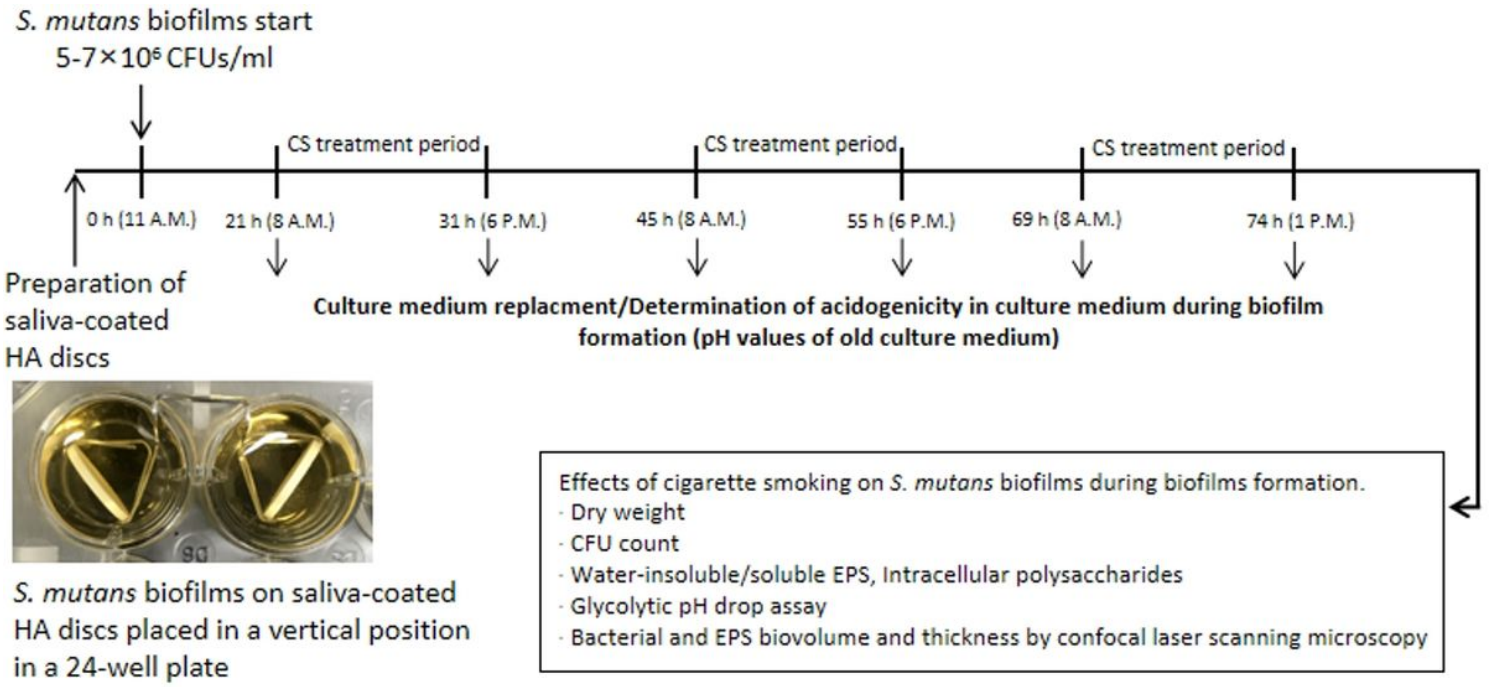


Figure 8

S. mutans biofilms formation and experimental scheme.

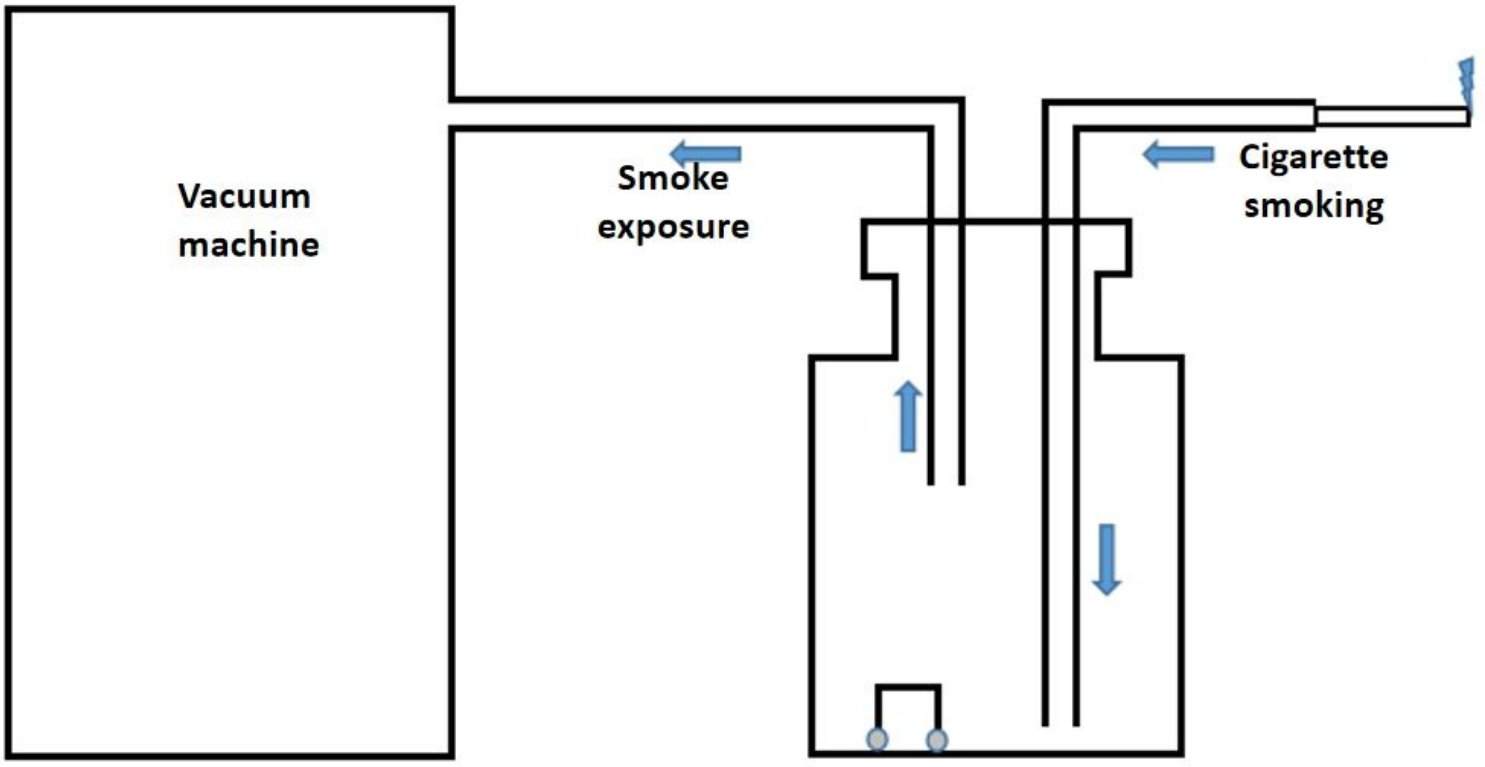


Figure 9

The smoking device.