

아저해농도(亞沮害濃度)의 항균물질이 mutans streptococci의 세포표면성질과 독력인자에 미치는 영향

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국문초록

아저해농도(亞沮害濃度)란 세포의 성장을 저해하는 최소 저해 농도(minimum inhibitory concentrations; MIC)이하의 농도를 말하는데, 적용된 화학물질의 동력학에 따라 항균물질은 반드시 이러한 농도로 존재하는 시기를 거치게 되며, 아저해농도의 항균물질은 세포의 성장을 저해하지는 않지만 세균의 대사와 성장에 스트레스로 작용하게 되어 세포의 성질과 독력인자 발현에 영향을 줄 수 있을 것이다.

본 연구는 여러 항균물질의 최소저해농도를 결정하고 아저해농도의 항균물질이 존재 시 세포 성질과 독력 인자에 미치는 영향을 알아보고, 치아우식증의 예방에 대한 화학적 접근법의 효용성을 조사하기 위해 계획되었다.

연구대상 세균으로는 mutans그룹 streptococci 중 두 가지 대표적인 세균종인 *S. mutans* (*S. mutans* Ingbritt)와 *S. sobrinus* (*S. sobrinus* 6715-7)를 사용하였고 화학물질 8종 (Sanguinaria extract; SG, Chlorhexidine digluconate; CHX, Fluoride; F, Propolis; PP, Hydrogen peroxide; HP, Triclosan; TC, Sodium dodecyl sulfate; SDS, Cetylpyridinium chloride; CC)을 단계 희석하여 배지에 첨가하여 최소저해농도를 구하였으며 아저해농도의 항균물질을 배지에 첨가하여 5% CO₂ 존재 하에 37℃에서 배양하여 이러한 항균물질을 첨가하지 않은 배지에서 배양한 세균과 성장속도, 산생성능, 체표소수성, 수산화인회석에의 부착능, glucan생성능, 세균응집능 등을 비교 관찰하였다.

아저해농도의 항균물질은 세포의 성장과 체표소수성, 타액으로 처리한 수산화인회석에 대한 세균의 부착과 glucan합성에 영향을 주는 것이 관찰되었다. 또한 12시간 배양 후 *S. mutans*와 *S. sobrinus* 모두에서 대조군에 비해 통계적으로 유의하게 최종 pH가 높게 나타나 산생성능이 억제되는 것이 관찰되었다 ($p < 0.05$). 세균을 proteinase K로 전처리한 경우 처리하지 않았을 때 보다 세균응집역가가 증가하거나 응집이 관찰되지 않았다.

본 연구의 결과에 따르면, 각각의 항균물질은 아저해농도에서도 알려진 작용기전과 유사하게 세균의 성질에 영향을 주었고 이러한 농도에서도 지속적으로 세균을 억제할 수 있었다. 따라서 이러한 항균물질의 사용은 치아우식증의 효과적인 예방법의 하나가 될 수 있다.

주요어 : 아저해농도, mutans streptococci, 세포표면성질, 독력인자

I. Introduction

Plaque formed by mutans streptococci plays a crucial role in the development and progression of dental caries and periodontal diseases. The removal of dental plaque would significantly reduce dental diseases. However, mechanical removal of plaque is limited. As adjunct methods of removing plaque, studies

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have been conducted on various methods including chemical approaches^{1,2)} and drugs having various antibacterial, antiplaque and anticariogenic effects³⁻¹⁹⁾, resulting in the detection of various chemicals having anticariogenic effects, which were applied in toothpaste, mouthwash, dental gel and dental varnish to prevent plaque formation inducing dental diseases²⁰⁾. Despite the fact that many studies were conducted on the effects and the mechanism of antimicrobial agents that prevent plaque formation, few study was conducted on the early stage of plaque formation, especially on the effects of these antimicrobial agents on the surface properties of bacteria and virulence factors such as the ability of bacteria attaching to the acquired pellicle on the tooth surface. The effects of antimicrobial agents preventing plaque formation depend on the concentration of the agent used, but the concentration of a chemical agent in the mouth is not constant. The antimicrobial agent used could exist in the concentration higher than the minimum inhibitory concentration (MIC) at the early stage but exists at lower concentrations than MIC as the agent decreases from the mouth by pharmacodynamics.

Sub-MICs (sub-MICs) refer to concentrations below MICs preventing cellular growth. Although cellular growth is not inhibited at sub-MICs, this concentration acts as stress to cellular metabolism and growth, affecting cellular properties and the expression of virulence factors²¹⁾.

The present study was conducted to determine the subinhibitory concentration (sub-MICs) of the cariogenic bacteria, mutans streptococci, and the effects of sub-MICs of antimicrobial agents on cellular properties and virulence factors, and to establish a model that could be used to evaluate continuously developing new substances and effectiveness of chemical approach for the prevention of dental caries.

II. Material and methods

The bacterial strains used for this study were *Streptococcus mutans* Ingbritt and *Streptococcus sobrinus* 6715-7, which were obtained from SNUCTC (Seoul National University Collection for Type Culture). Aliquots of frozen bacteria were grown overnight in 10 ml of Todd-Hewitt broth (THB, Difco, Detroit, Michigan, USA) at 37°C in 5% CO₂.

Sanguinaria (SG), Chlorhexidine digluconate (CHX), Fluoride (F), Propolis (PP), Hydrogen Peroxide (HP), Triclosan (TC), Sodium dodecyl sulfate (SDS) and Cetylpyridinium chloride (CC) were evaluated. All the agents were obtained from Sigma (St. Louis, Minneapolis, USA) except triclosan (Ciba Specialty Chemicals, Basel, Switzerland).

Each antimicrobial agent was serially diluted in THB to determine MICs and the optical density of the cultures was determined for 18 h. The MIC was defined as the lowest concentration of a chemical agent which inhibited bacterial growth upon reference to predetermined standard curves.

For the following assay, the sub-MIC of antimicrobial agents was selected as the highest concentration that enabled growth of the bacteria equivalent to an increase of the absorbance values at 660 nm equal to or higher than 0.04 after incubation for 10 h at 37°C. The cells that had been grown in the absence of antimicrobial agents were used as the controls.

The hexadecane assay described by Rosenberg et al.²²⁾ was used to determine the cell surface hydrophobicity. Growth rate was determined by the optical density at 660 nm for 12 h and compared with controls. The final pH of culture media was also determined by pH meter after observation of growth state.

The bacterial adherence was assayed by a modification of the method of Clark et al.²³⁾. The saliva from one donor was clarified by centrifugation at 8,000 × g for 15 min at 4°C, and the supernatant was heated to 60°C for 30 min to inactivate degradative enzymes. The bacterial inoculum was prepared in THB supplemented with [methyl-³H]-thymidine (Amersham, Little Chalfont, Buckinghamshire, UK) at a final concentration of 0.5 μCi/ml. The cells were gently sonicated for 2 min to break chains and cell densities were adjusted to a concentration of 1 × 10⁹ cells/ml.

Forty mg of Spheroidal hydroxyapatite (HA) beads (Biorad, Hercules, California, USA) beads were placed in polypropylene tubes and incubated with 1 ml of clarified saliva for 30 min at room temperature in an apparatus which continuously inverted the tubes ten times each minute. The beads were then washed twice with buffered KCL. Samples of 1 ml of ³H-labeled bacteria in buffered KCL were added to each tube of beads. After incubation for 2 h at room

temperature, 200 μ l samples of supernatant fluid were moved to scintillation vials to determine the number of free cells. The beads were washed twice in buffered KCL, dried and transferred to scintillation vials. Five ml of scintillation liquid was added to the vials. The numbers of cells per milliliter and of cells bound were measured by scintillation counter (Beckman, Fullerton, California, USA). The glucan synthesis was assayed as follows. The cells were washed with sodium phosphate buffer (PB, pH 7.0) and resuspended in the same buffer. Cell densities were adjusted to a concentration of 1×10^9 cells/ml. One ml of bacterial suspension was incubated with 100 mM sucrose at 37 °C for 12 h and harvested by centrifugation ($10,000 \times g$, 10 min). The supernatant was used to determine soluble glucan and the cells for insoluble glucan respectively by phenol-sulfate colorimetric method of Dubois et al.²⁴⁾

The cells cultured in 300 ml of THB and harvested by centrifugation. The cells were washed 3 times with saline and their optical density was adjusted to 3.0 at 550 nm. 50 μ l of the cell suspension and an equal volume of twofold serial dilution of the antimicrobial agents were mixed in 96 well plates and incubated at 37 °C for 2 h. The aggregation titer was expressed as the minimum concentration of antimicrobial agents to induce cellular aggregation. The whole cells of mutans streptococci were treated with protease as follows. Two hundred milliliters of cell suspension was incubated with 3 mg proteinase K (Merck, Darmstadt, Germany) for 18 h at 37 °C. The protease-treated cells were washed with saline and suspended to an optical density of 3.0 at 550 nm. The aggregation titer of the protease-treated cells was measured according to the method described above.

All the experiment were conducted in triplicate and all the assays were analyzed by paired t test for significant differences between antimicrobial agents and controls.

III. Results

Minimum inhibitory concentrations were expressed in mole concentration other than propolis (Table1). *S. sobrinus* showed the same or lower MICs compared with those of *S. mutans*, suggesting that *S. sobrinus* was more sensitive to antimicrobial agents.

In both cases of *S. mutans* Ingbritt and *S. sobrinus* 6715-7, growth inhibition was distinct with the addition of sanguinaria. Differences of more than 0.09 were seen in Groups F, CC and HP of *S. mutans* and in Group F, SDS and PP of *S. sobrinus* (Fig. 1).

Hydrophobicity was significantly decreased ($p < 0.05$) in *S. mutans* in all study groups compared with the control group. It was increased or decreased compared with the control group in *S. sobrinus* with Groups CHX, PP, and TC, showing no statistically significant difference with the control group and the rest of the groups showing significant differences with various increases and decreases (Fig. 2).

The pH of the culture without cell inoculation was 7.78. By 12 h of incubation, the culture media of *S. mutans* and *S. sobrinus* all showed statistically significantly high pHs compared with the control group, showing the inhibition of acid production by bacteria (Table 2).

In *S. mutans* Ingbritt, adhesion to HA treated with saliva increased in all groups compared with the control group. In *S. sobrinus* 6715-7, it decreased in all groups other than Group HP with statistical significance (Fig. 3).

Table 1. MICs. (Minimum inhibitory concentration) of antimicrobial agents

	<i>S. mutans</i> Ingbritt	<i>S. sobrinus</i> 6715-7
Sanguinaria	2.12×10^{-5} M	1.06×10^{-5} M
Chlorhexidine	3.91×10^{-7} M	7.81×10^{-7} M
Sodium fluoride	7.44×10^{-3} M	1.86×10^{-3} M
Propolis	0.0625 mg/ml	0.03125 mg/ml
Hydrogen peroxide	3.91×10^{-5} M	3.91×10^{-5} M
Triclosan	2.50×10^{-3} M	2.50×10^{-3} M
Sodium dodecyl sulfate	2.17×10^{-4} M	1.08×10^{-4} M
Cetylpyridinium chloride	1.15×10^{-5} M	1.15×10^{-5} M

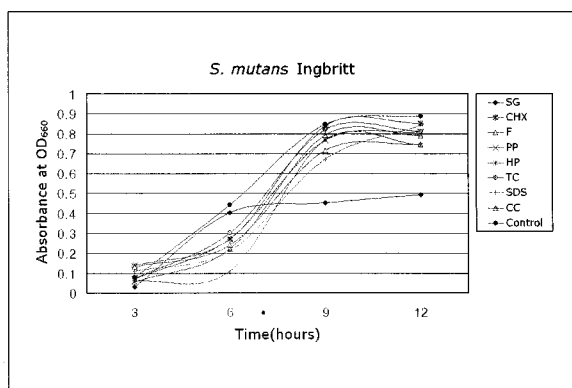


Fig. 1. Growth curve of *S. mutans* Ingbritt and *S. sobrinus* 6715-7 in the absence (control) or presence of sub-MICs of antimicrobial agents.

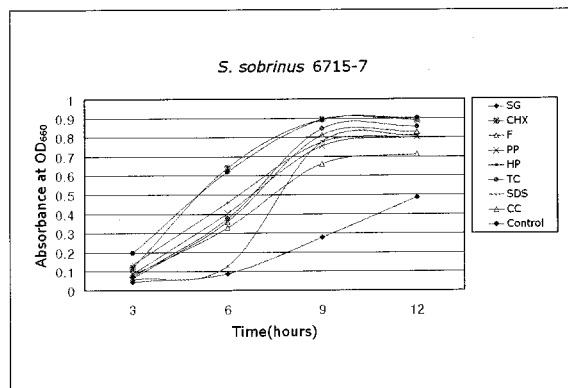


Fig. 2. Percentage of hydrophobicity of *S. mutans* Ingbritt and *S. sobrinus* 6715-7 in the absence (control) or presence of sub-MICs of antimicrobial agents.

SG; Sanguinaria, CHX; Chlorhexidine, F; Sodium fluoride, PP; Propolis, HP; Hydrogen peroxide, TC; Triclosan, SDS; sodium dodecyl sulfate, CC; Cetylpyridinium chloride group

Table 2. Final pH of media of *S. mutans* Ingbritt and *S. sobrinus* 6715-7 in the absence (control) or presence of sub-MICs of antimicrobial agents

	<i>S. mutans</i> Ingbritt	<i>S. sobrinus</i> 6715-7
SG	6.17±0.40	6.25±0.04
CHX	6.40±0.60	6.27±0.05
F	5.89±0.04	5.83±0.04
PP	5.95±0.01	5.79±0.06
HP	5.57±0.01	5.50±0.02
TC	5.67±0.01	5.49±0.02
SDS	5.63±0.01	5.44±0.07
CC	5.74±0.20	5.59±0.20
Control	5.02±0.10	4.99±0.10

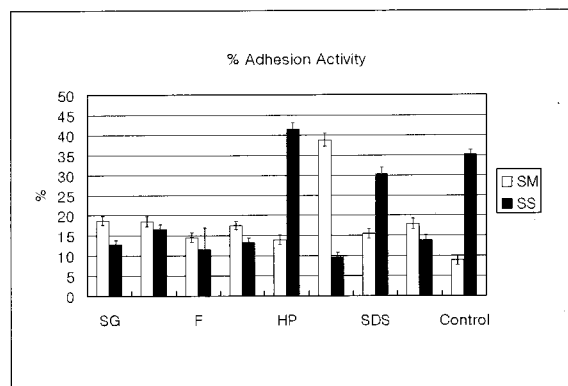
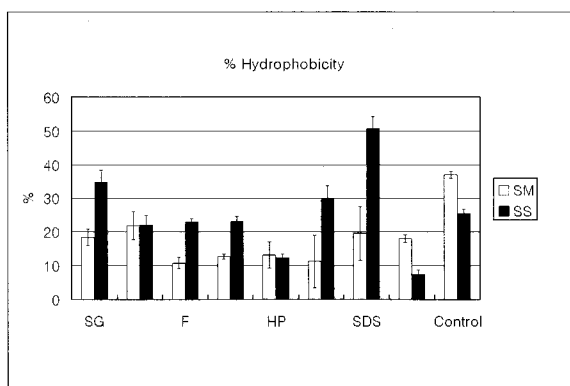


Fig. 3. Percentage adhesion to saliva-coated hydroxyapatite beads of *S. mutans* Ingbritt and *S. sobrinus* 6715-7 in the absence (control) or presence of sub-MICs of antimicrobial agents.

SG; Sanguinaria, CHX; Chlorhexidine, F; Sodium fluoride, PP; Propolis, HP; Hydrogen peroxide, TC; Triclosan, SDS; sodium dodecyl sulfate, CC; Cetylpyridinium chloride group, SM; *S. mutans* (control), SS; *S. sobrinus* (control)

Table 3. Insoluble and soluble glucan synthesized by *S. mutans* Ingbritt and *S. sobrinus* 6715-7 in the absence (control) or presence of sub-MICs of antimicrobial agents

Insoluble Glucan	<i>S. mutans</i> Ingbritt		<i>S. sobrinus</i> 6715-7	
	Average(μ g)	\pm SD	Average(μ g)	\pm SD
SG	14.23*	0.16	17.39*	0.29
CHX	15.07*	0.36	14.37*	1.66
F	14.12*	0.02	11.96*	0.06
PP	17.19	0.18	17.96*	0.74
HP	16.41*	0.13	14.46*	0.26
TC	13.57*	0.07	15.91*	0.46
SDS	11.22*	0.40	9.37*	0.88
CC	11.91*	0.04	11.05*	0.02
Control	17.32	0.2	20.35	1.12

Insoluble Glucan	<i>S. mutans</i> Ingbritt		<i>S. sobrinus</i> 6715-7	
	Average(μ g)	\pm SD	Average(μ g)	\pm SD
SG	14.12*	0.08	19.42	0.46
CHX	15.04*	0.06	15.12*	0.24
F	14.21*	0.07	11.94*	0.02
PP	16.09	0.64	15.58	3.54
HP	16.30	0.34	14.39*	0.06
TC	13.74*	0.07	15.41*	0.14
SDS	10.93*	0.01	8.59*	0.01
CC	11.87*	0.01	10.87*	0.02
Control	16.83	0.14	19.45	0.50

Asterisks (*) represents the value of statistically significant difference compared with control ($p < 0.05$)

Table 4. Effect of antimicrobial agents on cell aggregation of oral streptococci cells with (Proteinase Tx.) or without (No Tx.) the pretreatment of proteinase K

Cell aggregation titer	<i>S. mutans</i> Ingbritt		<i>S. sobrinus</i> 6715-7	
	No Tx.	Proteinase Tx.	No Tx.	Proteinase Tx.
SG	15.63 μ g/ml	NA	62.5 μ g/ml	NA
CHX	0.00%	NA	0.00%	NA
F	31.25 μ g/ml	NA	500 μ g/ml	NA
PP	62.50 μ g/ml	NA	250 μ g/ml	NA
HP	1.50%	NA	0.16%	NA
TC	33.33 μ g/ml	NA	1.56 μ g/ml	50 μ g/ml
SDS	15.63 μ g/ml	50000 μ g/ml	7.81 μ g/ml	50000 μ g/ml
CC	50 μ g/ml	50 μ g/ml	0.78 μ g/ml	NA

NA: not aggregated

SG: Sanguinaria, CHX: Chlorhexidine, F: Sodium fluoride, PP: Propolis, HP: Hydrogen peroxide, TC: Triclosan, SDS: sodium dodecyl sulfate, CC: Cetylpyridinium chloride group

As shown in Table 3, the synthesis of insoluble glucan showed significant differences in all groups other than Group PP, and the synthesis of soluble glucan was significantly different in all groups other than Groups PP, and HP. Also in *S. sobrinus*, the synthesis of insoluble glucan was decreased statisti-

cally significantly in all groups, but the synthesis of soluble glucan was not significantly different in Groups SG and PP.

Cell aggregation was observed at very low concentrations, in which cell aggregation was not observed in most cases pretreated with proteinase K and cell

aggregation titer was increased by several tens of folds (TC-*S. sobrinus*) or several thousands of folds (SDS). However, no difference was seen with CC in *S. mutans* (Table 4).

IV. Discussion

The MICs were in the similar ranges as reported by other authors^{3-5,8,10,11,14,17-19} and somewhat differences were probably due to differences in the types of antibiotics used, media, and initial concentrations at the time of dilution. The MICs observed in the present study were significantly lower than actual concentrations used clinically. Generally, the concentration of fluoride contained in toothpaste varies from 1,000 ppm to 1,500 ppm, and sodium dodecyl sulfate is usually used between 1-3%. Mouthwashes contain high concentrations of chlorhexidine at 0.12-0.2%, high enough to show antiseptic activities. Usually cetylpyridinium chloride is used at 0.05%, and sanguinaria extract at 0.03%, which could be 0.01% equivalent to sanguinarine chloride. Propolis is usually used at 1%, and 10% hydrogen peroxide is used to disinfect, and triclosan at 2%. Thus, we could expect that cariogenic bacteria could be exposed to sub-MICs of the antimicrobial agents used in the present study through mouthwash, gel, dental varnish, and toothpaste.

The growth rate was most significantly inhibited in *S. mutans* according to the standard growth curve between 4 to 8 h after inoculation and growth was inhibited in all groups with the addition of the sub-MICs of antimicrobial agents in the period.

Significant growth inhibition was also seen with *S. sobrinus* in sanguinaria and continued after 12 h of inoculation and even lasted until 24 h (Data not shown). The growth rate was significantly distinct in *S. sobrinus* between 3 to 6 h of inoculation and was reduced in all groups when sub-MICs of antimicrobial agents were added; showing bacteriostatic effects even if the concentration was lower than MICs.

Furthermore, the final pH of medium was higher in all groups compared with that in control group, due to the effects of acid production, which is the most important mechanism involved in the development of dental caries by the cariogenic bacteria. However, this result was seen in liquid media in the laboratory

environment so that it should be analyzed carefully due to differences with actual plaque environment.

The hydrophobicity of bacteria is one of the most important factors involved in the adhesion of cariogenic bacteria on tooth surface²⁵. The property of *S. mutans* attaching to hexadecane was statistically significantly decreased in all study groups compared with control group. It was lower in *S. sobrinus* compared with *S. mutans*, similar to the result that we found in our previous study with the hydrophobicity showing the value around 30%.²⁶ However, other authors reported the hydrophobicity of mutans streptococci to be between 60-80%¹⁶. It was mainly related with cell membrane surface proteins²⁷, probably due to the effect on the proteins on cell surfaces coming from the difference in the types of bacteria used and study methods.

Hydrophobicity has dual characteristics in which it could be considered to be an important virulence factors having the characteristics of both adhesion to host tissue and colonization, however, the more hydrophobic, bacteria would be attacked by host immune cells and phagocytosis easier.

The hydrophobicity of cariogenic bacteria has much more significance in which a study reported that the mutant strains of *S. mutans* and *S. sanguis* with their hydrophobic property artificially removed could not adhere to HA²⁷, suggesting that the hydrophobicity affects the ability of adhesion significantly.

The ability of adhesion to saliva treated HA (SHA) was increased in all groups with *S. mutans* compared with the control group. On the other hand, adhesion was statistically significantly decreased in all groups with *S. sobrinus* other than HP group. Although the results of the present study suggest that no relationship is present between hydrophobicity and adhesion to SHA, the possibility still remains that hydrophobicity plays a crucial role in stabilizing the bonding between adhesins of bacteria and their receptors.

Actually, various adhesins of bacteria participate in the process of adhesion of bacteria on tooth surface²⁸⁻³⁰ and are related with various specific receptors present in tooth surface^{1,31}. Therefore, one could not explain adhesion only with hydrophobicity³². Antimicrobial agents may play a contrasting role against the surface proteins inducing bacterial adhesion and hydrophobicity. However, adhesion to HA

was increased in all study groups with *S. sobrinus* but the hydrophobicity was either increased or decreased. Therefore, the surface proteins participating in hydrophobicity could be different.

No cell aggregation was observed in SG, CHX, F and PP groups pretreated with proteinase in the present study. Cell aggregation titer was increased in all the rest groups pretreated with proteinase. Therefore, protein molecules present on bacterial surface would play an important role in the induction of cell aggregation by antimicrobial agents³³. The increase and loss of cell aggregation titer would probably express the degree of bonding between these antimicrobial agents with cell surface proteins. In addition, cell surface proteins would play a role in hydrophobicity and adhesion to saliva-coated HA. However, the contrasting results were obtained probably because the types and structures of proteins were different and bonding of antimicrobial agents to proteins and the effects of these agents on these proteins were also different.

Sucrose plays an essential role in colonization of mutans streptococci and cariogenicity by being synthesized into insoluble glucan by glucosyltransferase (GTF). The synthesis of both insoluble and soluble glucan was decreased in *S. mutans*, and the synthesis of insoluble glucan was decreased significantly in all groups other than PP group in the present study. One interesting finding was an increased synthesis of soluble and insoluble glucan in propolis group, and a study reported that propolis at high concentrations would inhibit cellular growth and metabolism but would not affect at all and rather increase them at low concentrations¹¹.

Insoluble glucan possesses more ligands than soluble glucan, acts as glue in mutans streptococci, and significantly related with bacterial aggregation or plaque maintenance. Nonetheless, since mutans streptococci were cultured and used after being washed to examine the synthesis of glucan, the amount of glucan measured in the present study was probably came only from cell bound GTF rather than total GTF or extracellular free GTF. Furthermore, bacteria were allowed to use sufficient sucrose by adding 100 mM of sucrose at the time of culturing and the consideration should given to the fact that the oral environment would differ than the laborato-

ry environment used in the present study.

The results varied in the this study probably because antimicrobial agents at sub-MICs inhibited the synthesis of structural component or proteins, changing the properties of cell surface at the time of cellular growth and antimicrobial agents at low concentrations directly affected the action site, changing the properties of cell surface and virulence.

The action mechanisms of various antimicrobial agents have not been elucidated but many different hypotheses exist. Thus, the exact mechanism of antimicrobial agents at sub-MICs on the virulence factors of mutans streptococci could not be determined.

The main action mechanism involved with sanguinarine is in the prevention of bacteria from attaching to the tooth membrane. Adhesion to HA treated with saliva was decreased in *S. sobrinus* and the synthesis of insoluble glucan was also decreased. On the other hand, the hydrophobicity of *S. mutans* was decreased in sanguinaria group and the synthesis of insoluble glucan was also decreased, suggesting that two action mechanisms were involved in the inhibition of adhesion with hydrophobicity having more influence on the inhibition of adhesion. Furthermore, the inhibition of growth was distinct, and this bacteriostatic effect was clinically very significant since sanguinaria has the substantivity at high concentrations within plaque, having the potential to inhibit colonization and growth of cariogenic bacteria.

It was reported that chlorhexidine could inhibit acid production of bacteria by the inhibition of the phosphoenol-pyruvate-mediated phosphotransferase (PTS) sugar transport system both *in vivo* and *in vitro*³⁴, and the final pH was the highest in chlorhexidine group compared with the other groups in this study. Moreover, another study reported it inhibits the adhesion by inhibiting enzymes related with the glycolysis³⁵, and the hydrophobicity was decreased and the synthesis of insoluble glucan was inhibited in the present study. It is known that chlorhexidine at high concentrations would destroy the cell membrane and induce the coagulation of cytoplasm by protein cross-linkage³⁵. Cellular destruction was not observed in the present study at the sub-MICs used since the antimicrobial agents used were diluted by 50-100 folds from 0.12-0.2%, the clinically used concentrations.

The similar cationic cetylpyridinium chloride also showed the similar antimicrobial spectrum as chlorhexidine, and it is known that the main mechanism involved is the inhibition of cell adhesion, glycolytic enzyme, and sugar metabolism³⁶⁾. In the present study the synthesis of insoluble glucan was significantly decreased in CC group, and the hydrophobicity was also decreased in *S. mutans* and *S. sobrinus*. Growth inhibition along with higher final pH compared with the control group was observed, showing that cariogenicity could be decreased by affecting all virulence factors investigated other than increased adhesion to saliva-treated HA in *S. mutans*.

Sodium dodecyl sulfate is known to have a strong affinity for protein molecules and strong denaturing effect, inhibiting bacterial adhesion³⁷⁾. Furthermore, this antimicrobial agent could specifically inhibit enzyme action even at sub-MICs, such as GTF in *S. mutans* or PTS in *S. sobrinus* for example³⁷⁾. The synthesis of both soluble and insoluble glucan resulted by these enzymes was decreased in the present study. One characteristic was the fact that both the hydrophobicity and adhesion to saliva-treated HA were increased in *S. sobrinus*. This result suggests the possibility that sodium dodecyl sulfate induced changes in surface proteins related with adhesion in *S. sobrinus*, facilitating adhesion. And further studies are needed on this subject.

Many studies were conducted on the action mechanisms of anionic fluoride and various mechanisms reported involved an increase in acid resistance of enamel, inhibition of glycolysis enzymes, and inhibition of acidogenesis in bacteria present within plaque³⁴⁾. Other than the actions by host factors such as the induction of remineralization and increased aciduricity among these mechanisms, the synthesis of both type of glucan was decreased in the present study. Furthermore, the hydrophobicity was decreased significantly in fluoride group, and adhesion to HA was significantly decreased in *S. sobrinus*, showing also the effect on bacterial adhesion.

The non-ionic triclosan is known to inhibit bacteria from taking glucose by inhibiting PTS and Proton motive force³⁸⁾. Also in the present study, the synthesis of both soluble and insoluble glucan was also decreased in all groups. The hydrophobicity was de-

creased in *S. mutans*, whereas adhesion to HA was characteristically increased significantly. On the other hand, no difference was seen in hydrophobicity in *S. sobrinus* and adhesion to HA was decreased significantly. As mentioned earlier, it is believed that contrasting results were seen because the proteins participating in adhesion showed contrasting actions, requiring further studies on this matter.

Hydrogen peroxide has been traditionally used as a disinfectant with the main action mechanism involving the germicidal action due to free radicals and a cleansing action due to bubble formation³⁹⁾. In the present study, although the synthesis of insoluble glucan was decreased, the degree of decrease was least significant among different study groups. Adhesion to HA was increased in both *S. mutans* and *S. sobrinus*; thus, the main action mechanism involved was probably mechanical removal of plaque rather than the effect on the virulence factors. However, hydrogen peroxide would be clinically effective in areas where mechanical removal of plaque would be difficult by using bubbles to remove plaque since the hydrophobicity was characteristically significantly decreased in *S. mutans* and *S. sobrinus*.

In short, MICs of antimicrobial agents were significantly lower than those concentrations used in actual clinical settings. Antimicrobial agents at sub-MICs decreased the growth rate and acid production, and changed various virulence factors such as the degree of adhesion to saliva-coated HA, glucan synthesis and hydrophobicity of the cariogenic bacteria. These results varied probably because antimicrobial agents at sub-MICs changed the characteristics of cell surface by inhibiting certain structural components or protein synthesis during cell growth and antimicrobial agents at low concentrations changed the characteristics of cell surface and virulence factors by directly acting on the cell and its action site. Furthermore, antimicrobial agents actually exist in the mouth at sub-MICs and the presence of these agents at these concentrations would affect the pathogenesis of dental caries caused by cariogenic bacteria in various ways. According to the results of the present study, each antimicrobial agent at sub-MICs could affect similar as its known action mechanism and could continually inhibit cariogenic bacteria at sub-MICs. Thus, the use of these antimicrobial agents

would be one of the effective methods to prevent dental caries.

Future studies need to include not only the effects of sub-MICs of antimicrobial agents and but also effects on interaction between plaque being the cause of dental caries and bacteria within plaque. Furthermore, it would be useful to apply the study model used in the present study in order to verify new drugs having anticariogenic effects and anti-plaque effects. And further experimental studies would be needed on the pathogenesis of bacteria inducing diseases and virulence factors, along with the verification of effectiveness through clinical studies.

V. Conclusion

Eight chemical agents (Sanguinaria extract: SG, Chlorhexidine digluconate: CHX, Fluoride: F, Propolis: PP, Hydrogen Peroxide: HP, Triclosan: TC, Sodium dodecyl sulfate: SDS, Cetylpyridinium chloride: CC) were diluted serially in broth of *Streptococcus mutans* (*S. mutans* Ingbritt) and *Streptococcus sobrinus* (*S. sobrinus* 6715-7) to determine MICs and compared the growth rate, acid production, hydrophobicity, adhesion activity to saliva coated hydroxyapatite, glucan synthesis and cellular aggregation of experiment groups (in the presence of sub-MICs) with those of control (in the absence of antimicrobial agent).

The obtained results were as follows:

1. Determination of the MICs showed that *S. sobrinus* was more susceptible to all the agents.
2. Growth of cells in EM and SG was inhibited and it was recovered 6 hours after inoculation in the other groups.
3. Hydrophobicity was reduced in all the *S. mutans* groups, especially in antibiotics groups, but reduced or increased in *S. sobrinus*.
4. Subinhibitory concentrations of antimicrobial agents resulted in a significant reduction in their pH after 12 hours ($p < 0.05$).
5. The adhesion of bacteria to saliva coated hydroxyapatite was increased in all the groups of *S. mutans* but decreased in *S. sobrinus* except HP ($p < 0.05$).
6. Insoluble glucan synthesis was decreased significantly in all the groups of *S. mutans* except PP and all the groups of *S. sobrinus* ($p < 0.05$). Soluble glucan synthesis was decreased significantly in all the groups except PCN, PP, HP of *S. mutans* and SG, PP of *S. sobrinus* ($p < 0.05$).
7. By pretreating cells with proteinase K, the aggregation induced by antimicrobial agents was completely inhibited or the aggregation titers were markedly increased.

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Abstract

EFFECTS OF SUBINHIBITORY CONCENTRATIONS OF ANTIMICROBIAL AGENTS ON CELL SURFACE PROPERTIES AND VIRULENCE FACTORS OF MUTANS STREPTOCOCCI

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Subinhibitory concentrations (sub-MICs) refer to concentrations below minimum inhibitory concentrations (MICs). The antimicrobial agents may be present at relatively high concentration, at least higher than bacterial MIC and thereafter be desorbed off a surface and function at sub-MICs, perhaps by interfering with bacterial metabolism. Consequently, the aim of this study was to determine the effects of growth, in the presence of sub-MICs of antimicrobial agents, on the cell surface properties and virulence factors of mutans streptococci and to investigate the efficacy of a chemical approach *in vitro*.

Streptococcus mutans Ingbritt and *Streptococcus sobrinus* 6715-7 were used. Eight antimicrobial agents (Sanguinaria extract:SG, Chlorhexidine digluconate:CHX, Fluoride:F, Propolis:PP, Hydrogen peroxide:HP, Triclosan:TC, Sodium dodecyl sulfate:SDS, Cetylpyridinium chloride:CC) were diluted serially in broth to determine MICs and to compare the growth rate, acid production, hydrophobicity, adhesion activity to saliva coated hydroxyapatite, glucan synthesis and cellular aggregation of experiment groups (in the presence of sub-MICs) with those of control (in the absence of antimicrobial agents).

Sub-MICs of antimicrobial agents affected the growth of cells, hydrophobicity, and adhesion of bacteria to saliva coated hydroxyapatite and glucan synthesis. They also resulted in a significant reduction in pH after 12 hours ($p < 0.05$). By cells pretreated with proteinase K, either the aggregation induced by antimicrobial agents was completely inhibited or the aggregation titers were markedly increased.

According to the results of the present study, each antimicrobial agent at sub-MICs could affect similar as its known action mechanism and could continually inhibit cariogenic bacteria at such concentrations. Thus, the use of these antimicrobial agents would be one of the effective methods to prevent dental caries.

Key words : Subinhibitory concentration, Mutans streptococci, Cell surface properties, Virulence factors