Note

Antimicrobial Activity of Protamine against Oral Microorganisms

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Protamine is an arginine-rich polycationic protein extracted from sperm cells of vertebrates including fishes such as salmon. The purpose of this study was to investigate the suppressive effects of protamine on the growth of oral pathogens for possible usage in dental materials. Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) were determined by the microdilution method. Twelve strains of oral viridans streptococci, *Actinomyces naeslundii*, *Actinomyces odontolyticus*, *Enterococcus faecalis*, *Lactobacillus acidophilus*, *Aggregatibacter actinomycetemcomitans*, *Fusobacterium nucleatum*, *Porphyromonas gingivalis* and *Candida albicans* were suppressed by protamine. MIC and MBC values were between 0.009~20 mg/mL and 0.019~80 mg/mL, respectively. The bactericidal activities of protamine against susceptible bacterial species were dependent on the concentration of protamine and incubation time. Based on the results of this study, protamine would be a useful compound for the development of antimicrobial agents against oral pathogens in dental materials.

Key words : Antimicrobial agent / Bacteria / Protamine / Salmon.

The emergence of antibiotic-resistant bacterial strains has led to an increased attempt to develop alternative antimicrobial agents (Baltzer and Brown, 2011). Recently, nonantibiotic compounds such as antibacterial peptides have been widely investigated. Different kinds of small cationic peptide with broad-spectrum antibacterial activity such as defensin NP-1 and magainin 1 play an important role in the innate immune system of various animal species (Cederlund et al., 2011).

Protamine is a series of arginine-rich cationic polypeptides extracted from the sperm cells of vertebrates including fishes such as a salmon (Ando et al., 1973). Protamine is used in medicine as an antidote to heparin (Jaques, 1973) and as a carrier of insulin (Owens, 2011). Protamine/DNA complex has recently also been reported to be used for an injectable dental

material (Fukushima et al., 2011). It has been reported that protamine has antimicrobial properties against a wide range of bacteria and yeasts. For example, *Bacillus subtilis* (Antohi and Popescu, 1979), *Salmonella* Typhimurium (Aspedon and Groisman, 1996), *Escherichia coli* (Hansen and Gill, 2000) and *Candida utilis* (Yphantis et al., 1967) have been shown to be sensitive to protamine. Johansen et al. (1995) reported that protamine from salmon killed Gram-positive bacteria and significantly inhibited the growth of Gram-negative bacteria. Richards et al. (1990) reported that protamine depressed the metabolic activity of *Staphylococcus epidermidis* in a biofilm.

Oral bacterial species cause several different types of diseases. For example, mutans group streptococci are known as etiological bacterial species for dental caries (Hamada and Slade, 1980) and some Gram-negative anaerobic bacteria have been suggested to cause periodontal diseases (Haffajee and Socransky, 1994). Even though protamine has antimicrobial activities

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against some oral microbial species such as *Porphyromonas gingivalis*, *Prevotella intermedia*, *Aggregatibacter actinomycetemcomitans* and *Candida albicans* (Miura et al., 2010a; Miura et al., 2010b), it is rare to find studies on the antimicrobial activity of protamine against oral microorganisms. The purpose of this study was to investigate the antimicrobial effect of protamine on a wide range of oral microbial species in consideration of its possible usage in dental materials.

Streptococcus gordonii DL1, *Streptococcus rattus* BHT, *Streptococcus mutans* ATCC 25175, *Streptococcus sobrinus* 6715 and *Streptococcus sanguinis* ATCC 10556 were laboratory strains stocked in Department of Microbiology and Immunology, College of Dentistry, Gangneung-Wonju National University. *S. mutans* KN405, *S. sanguinis* KN420, *Streptococcus anginosus* KN427, *Streptococcus intermedius* KN433, *Streptococcus constellatus* KN436, *Streptococcus salivarius* KN440 and *Streptococcus oralis* KN444 were isolated strains from Korean dental plaque and stocked in the same laboratory. *Actinomyces naeslundii* CCUG 35333T, *Actinomyces odontolyticus* ATCC 17929, *C. albicans* KCTC 7270, *Enterococcus faecalis* KCTC 3206, *E. faecalis* KCTC 2011, *Lactobacillus acidophilus* ATCC 4355, *A. actinomycetemcomitans* ATCC 33384, *Fusobacterium nucleatum* ATCC 23726 and *P. gingivalis* ATCC 33277 were obtained from the Korean Collection for Oral Microbiology (Chosun University, Gwangju, Korea).

Oral streptococci were grown in brain heart infusion broth (BHI) (Becton, Dickinson and Company, Sparks, MD, USA) for 18 h at 37°C in aerobic conditions supplemented with 5% CO₂. *Actinomyces* were cultured in BHI for 18 h at 37°C under anaerobic conditions (Bactron Anaerobic Chamber, Sheldon Manufacturing Inc. Cornelius, Oregon, USA) with an atmosphere of 90% N₂, 5% CO₂ and 5% H₂. *Lactobacillus* were grown in lactobacillus MRS medium (Becton, Dickinson and Company, Sparks, MD, USA) for 18 h at 37°C in aerobic conditions supplemented with 5% CO₂, and enterococcus were grown in BHI for 18 h at 37°C under aerobic conditions. *A. actinomycetemcomitans* was inoculated in BHI broth and *F. nucleatum* and *P. gingivalis* were cultured in prereduced trypticase soy broth (Becton, Dickinson and Company) containing 1 mg/mL of yeast extract (Becton, Dickinson and Company), 5 µg/mL of hemin (Sigma Chemical Co., St. Louis, MO, USA) and 1 µg/mL of menadione (Sigma Chemicals Co.). These three species were incubated in anaerobic conditions (Bactron Anaerobic Chamber) with an atmosphere of 90% N₂, 5% CO₂ and 5% H₂. Sabouraud Dextrose medium (Becton, Dickinson and Company) was used for culturing *C. albicans* which was incubated for 18 h at 25°C under aerobic

conditions. The culture media supplemented with bacteriological agar were used for solid agar plates.

To determine the MIC of protamine, a stock solution of protamine sulfate from salmon (Sigma-Aldrich Chemical Co.) was prepared in microbial culture medium for each microbial species (final concentration 80 mg/mL). Stock solution was filter sterilized. The MICs were determined by a microdilution method in culture medium which was modified from methods for antimicrobial susceptibility tests of the Clinical and Laboratory Standards Institute (CLSI) (2009). Using a microbial culture in the late log phase or stationary phase, a suspension equivalent to that of approximately 1×10^8 CFU/mL in each microbial culture medium was prepared. The bacteria were inoculated into serially diluted protamine solutions in 96-well round bottom microtitration plates for final concentrations of 5×10^5 CFU/mL. The final volume was 100 µL in a microtitration plate well. The microdilution trays were incubated in the same conditions described in bacterial culture conditions. MICs of protamine against aerobic and anaerobic bacteria were determined after incubation for 18 and 48 h, respectively. The microtitration plates were read visually and the minimum concentration of protamine showing no turbidity was determined as the MIC. MBC of protamine was determined by taking a 100 µL culture medium of each

TABLE 1. MIC and MBC of protamine against oral microorganisms.

Microorganism	Protamine	
	MIC ^a	MBC ^a
<i>S. anginosus</i> KN427	5	20
<i>S. constellatus</i> KN436	5	10
<i>S. gordonii</i> DL1	0.625	0.625
<i>S. intermedius</i> KN433	1.25	1.25
<i>S. mutans</i> ATCC 25175	0.009	0.019
<i>S. mutans</i> KN405	0.078	0.156
<i>S. oralis</i> KN444	1.25	1.25
<i>S. rattus</i> BHT	5	20
<i>S. salivarius</i> KN440	0.625	1.25
<i>S. sanguinis</i> ATCC 10556	1.25	2.5
<i>S. sanguinis</i> KN420	1.25	2.5
<i>S. sobrinus</i> 6715	0.039	0.625
<i>A. naeslundii</i> CCUG 35333T	1.25	10
<i>A. odontolyticus</i> ATCC 17929	0.625	1.25
<i>L. acidophilus</i> ATCC 4355	2.5	10
<i>E. faecalis</i> KCTC 3206	2.5	40
<i>E. faecalis</i> KCTC 2011	2.5	80
<i>A. actinomycetemcomitans</i> ATCC 33384	20	40
<i>F. nucleatum</i> ATCC 23726	5	5
<i>P. gingivalis</i> ATCC 33277	5	10
<i>C. albicans</i> KCTC 7270	1.25	5

^amg/mL.

bacterial species from the wells showing no visible growth and spreading it onto agar plates. The agar plates were incubated for 3 days for aerobic bacteria and 7 days for anaerobic bacteria. The MBC was defined as the minimum concentration of protamine that showed $\geq 99.9\%$ reduction of the original inoculums. The experiments for determining MIC and MBC were repeated at least three times. We checked the MIC and MBC of chlorhexidine (Sigma-Aldrich Chemical Co.) as positive controls.

Among bacterial species tested in this study, *S. mutans* KN405, *S. sobrinus* 6715, *L. acidophilus* ATCC 4355 and *A. odontolyticus* ATCC 17929, which were known to be related to the initiation and progress of dental caries, were selected for the further experiments. For examining the effects of concentrations of protamine, serially diluted protamine was added in wells containing bacteria and then incubated for 1 h. In another set of experiments, the effect of incubation time on the killing of bacteria by protamine was investigated by increasing the incubation time at the final protamine concentration of 10 mg/mL. Each experimental group consisted of duplicate wells. After incubation, each sample was serially diluted with PBS and 100 μ L of diluents was spread on duplicate agar plates for each species of microorganism. The plates were incubated for 72 h at 37°C for each species and then the number of CFU was determined by an automatic colony counter (IUL, Barcelona, Spain).

For examining the effect of protamine on the bacterial surface structure, *S. mutans*, one of the major etiological agents for inducing dental caries, was used. Variable pressure field emission scanning electron microscopy (VP-FE-SEM) SUPRA55VP (Carl Zeiss, Oberkochen, Germany) was used to examine the morphological changes in the *S. mutans* KN405. The bacteria were treated with 1 mg/mL of protamine for 4 h at 37°C and then centrifuged at 10,000 $\times$ g. Bacterial pellets were fixed in 2.5% glutaraldehyde in PBS (pH 7.4) for 1 h at room temperature. The fixed samples were then washed three times with PBS for 10 min and dehydrated for 30 min in a graded series of ethanol. After critical point drying, the samples were mounted on stubs, coated with gold and observed with SEM.

The Kruskal-Wallis test was used to compare the effects of the incubation time and the protamine concentration on bacterial killing. The level of significance was $P < 0.05$. Correlations between bacteria killing and the incubation time or protamine concentration were evaluated by calculating Spearman's rank correlation coefficient (Spearman's rho). The statistical analysis was performed using the Software Package for Social Sciences (SPSS, version 20.0, IBM Inc., USA).

Twelve strains of oral viridans streptococci, *A. naeslundii*, *A. odontolyticus*, *E. faecalis*, *L. acidophilus*, *A. actinomycetemcomitans*, *F. nucleatum*, *P. gingivalis* and *C. albicans* were used in this study. The MIC of protamine against oral streptococci was lower than 5 mg/mL. MICs for *A. naeslundii* and *A. odontolyticus* were 1.25 and 0.625 mg/mL, respectively. The MIC for lactobacillus was 2.5 mg/mL and MICs for periodontopathogens such as *P. gingivalis* and *F. nucleatum* were 5 mg/mL. The MIC for *S. mutans* ATCC 25175 was the lowest (0.009 mg/mL) followed by those for *S. sobrinus* 6715 (0.039 mg/mL) and *S. mutans* strain KN405 (0.078 mg/mL) in that order. *A. actinomycetemcomitans* showed the highest MIC (20 mg/mL) among the microbial strains examined in this experiment. *C. albicans*, the only eukaryotic cell used in this study, also showed a low MIC (1.25 mg/mL) comparable to oral bacterial strains. The MIC and MBC values of chlorhexidine for the bacterial strains used in this study were 0.001 ~ 15.6 μ g/mL and 0.001 ~ 62.5 μ g/mL, respectively. These values were similar to the values for oral bacteria reported in another study (Emilson, 1977).

The effects of protamine concentration and incubation time on the bactericidal activity against oral bacteria were examined by using *S. mutans* KN405, *S. sobrinus* 6715, *L. acidophilus* ATCC 4355 and *A. odontolyticus* ATCC 17929. The bactericidal activities of protamine depended on the concentration of protamine and incubation time (Fig. 1a-d). Incubation with protamine for 90 min at 10 mg/mL inhibited the growth of *S. mutans* KN405 by 61% (Fig. 1a). Protamine also suppressed the growth of *A. odontolyticus* by 85% after incubation for 90 min (Fig. 1d). The difference in the CFU values as a function of the incubation time with protamine was statistically significant for all species of bacteria used in this study ($P < 0.05$) (Fig. 1). There were significant negative correlations between the CFU values and the incubation time for *S. mutans* ($R = -0.715$, $P < 0.01$), *S. sobrinus* ($R = -0.922$, $P < 0.01$), *L. acidophilus* ($R = -0.723$, $P < 0.01$) and *A. odontolyticus* ($R = -0.946$, $P < 0.01$). At 1.25 and 10 mg/mL of protamine, the number of *S. mutans* cells was reduced by 42 and 68%, respectively (Fig. 1a). Protamine at 1.25 and 10 mg/mL suppressed the growth of *S. sobrinus* by 30 and 53%, respectively (Fig. 1b). Protamine killed *A. odontolyticus* by 98% at 5 mg/mL (Fig. 1d). The difference in the CFU values as a function of the concentration of protamine was statistically significant for four species of bacteria used in this study ($P < 0.05$) (Fig. 1). The CFU values of *S. mutans* ($R = -0.892$, $P < 0.01$), *S. sobrinus* ($R = -0.896$, $P < 0.01$) and *A. odontolyticus* ($R = -0.946$, $P < 0.01$) had significant negative correlations with the

FIG. 1. Effects of the concentration of protamine and incubation time on the killing of bacteria. Values indicate means of duplicate determinations; standard deviations of the mean (error bars) are indicated by vertical lines.

concentration of protamine. The number of *L. acidophilus* CFUs and the concentration of protamine were not negatively correlated in a significant way ($R = -0.344$, $P = 0.138$).

S. mutans KN405 was observed by SEM after treatment with 1 mg/mL of protamine for 4 h at 37°C (Fig. 2b). The protamine treatment modified the cell surface texture of the bacteria. Small bumps appeared on the surface of *S. mutans* after treatment with protamine.

In this study, oral microbial species related to oral diseases and frequently discovered from dental plaque

FIG. 2. Scanning electron microscopy (SEM). (a) untreated bacteria, (b) bacteria treated with protamine.

were selected for the determination of the antimicrobial activity of protamine. *S. mutans* and *S. sobrinus* are known as causative agents for dental caries and lactobacilli also play a role in the progress of dental caries (Hamada and Slade, 1980). We examined some mitis group streptococci such as *S. sanguinis* and *S. gordonii* as initial colonizers in dental plaque formation (Coykendall, 1989). *Actinomyces* are found at root caries lesions (Bowden, 1990) and *E. faecalis* is related to endodontic infections (Kayaoglu and Orstavik, 2004). We also examined bacterial species, such as *Aggregatibacter*, *Fusobacterium* and *Porphyromonas*, which were suggested as causative agents for periodontal diseases. In this study, protamine had antibacterial activity against laboratory strains as well as freshly isolated strains. The MICs for mutans group streptococci, causative agents for dental caries, were much lower than those for other species. On the other hand, MICs of protamine against periodonto-pathogens such as *P. gingivalis*, *F. nucleatum* and *A. aggregatibacter* were higher than those against other oral species. In this study, the growth of *A. actinomycetemcomitans* appeared to be less affected by protamine in our susceptibility test. At present, we do not know what factors may contribute to protamine having a higher MIC than against other strains of oral bacteria. Interestingly, higher MICs and MBCs of antibacterial decapeptides against *A. actinomycetemcomitans* were reported than against other species of oral bacteria (Concannon et al., 2003).

The antibacterial effect of most polycationic peptides is generally related to their ability to disrupt the bacterial cell wall (Vaara, 1992). The action mechanism of the antimicrobial activity of protamine is suggested to be the electrostatic attraction between the positively charged protamine molecule and the negatively charged cell envelope, causing the inhibition or killing of bacteria due to the leakage of K^+ , ATP and intracellular enzymes (Johansen et al., 1997; Stumpe and Bakker, 1997). Another study indicated that protamine disrupted energy transduction and nutrient uptake functions, and

suggested that the cytoplasmic membrane was the target of protamine action (Aspedon and Groisman, 1996). However, all possible antibacterial mechanisms of protamine have not been disclosed yet and the killing mechanism of protamine against bacteria has been suggested to be different among bacterial species (Johansen et al., 1996). In the present study, changes in the surface of bacteria were observed by SEM after treatment with protamine. The bacterial surface change might be related to the mechanisms underlying the bactericidal effects of protamine. However, the morphological alteration of bacterial surfaces by protamine treatment was not very dramatic in this study.

Darouiche et al. (2008) reported that the combination of chlorhexidine and protamine had a synergistic inhibitory effect on *E. coli* and there was also a significant synergistic antibiofilm and antimicrobial activity against *E. coli*, *Pseudomonas aeruginosa* and *S. epidermidis*. The combination of protamine and chlorhexidine could be applied for the induction of synergistic antibacterial effects against oral bacterial species. Chlorhexidine has broad-spectrum antibacterial activity where its inhibition mechanism is partly from the disruption of the bacterial plasma membrane (Kuyyakanond and Quesnel, 1992). Protamine can increase the permeability of bacterial cell membrane, thereby facilitating the transport of chlorhexidine into the cytoplasm (Antohi and Popescu, 1979; Aspedon and Groisman, 1996).

The effect of protamine on the formation of bacterial biofilms was reported previously (Richards et al., 1990; Soboh et al., 1995). The combination of protamine with rifampin against *S. epidermidis* biofilms demonstrated synergism and showed a totally bactericidal outcome (Richards et al., 1990). Soboh et al. (1995) reported that the synergism between ciprofloxacin and protamine significantly enhanced the efficacy of ciprofloxacin against planktonic bacteria and biofilm caused by *P. aeruginosa*. The present study tested the antimicrobial effects of protamine against planktonic bacteria rather than biofilm bacteria. Thus, the antibacterial effects of protamine on bacterial biofilms might differ from those obtained in this study using planktonic bacteria. Therefore, further studies are required to investigate the suppressive effects of protamine against oral biofilms.

Recently, Miura et al. (2010a; 2010b) reported the antibacterial and antifungal activity of protamine against oral microorganisms, where there was inhibition on the growth of *P. gingivalis*, *P. intermedia* and *A. actinomycetemcomitans* and on the attachment of *C. albicans* to polymethyl methacrylate. The MIC of protamine for *P. gingivalis* was lower than that in this study. It is assumed that the discrepancy between MICs of these studies might result from the differences in the

method for determining MICs. They used agar plates containing protamine for determining MIC instead of the microdilution methods used in our study. Fukushima et al. (2010; 2011) developed a DNA/protamine complex paste for an injectable dental material. The antimicrobial activities of protamine against a wide variety of oral bacteria including cariogenic and periodontopathic bacteria in the present study may add advantages to the DNA/protamine-injectable dental material. Interestingly, protamine has been reported to inhibit the proteolytic activity of arginine-specific cysteine protease from *P. gingivalis* (Kontani et al., 1999). Protamine was also capable of binding strongly to *P. gingivalis* fimbriae and inhibited fimbrial interaction with immobilized fibronectin, which resulted in inhibiting the binding of *P. gingivalis* to host the surface (Kontani et al., 1999). These inhibitory characteristics of protamine against the virulence factors of *P. gingivalis* for inducing periodontal diseases would play a helpful role when protamine is added to dental care products.

In this study, protamine from salmon had antimicrobial activity against a wide range of oral pathogenic microbial species. The bactericidal activities of protamine against susceptible bacterial species were dependent on the concentration of protamine and incubation time. These findings might facilitate the possibility of using protamine in dental materials.

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