Synergistic Antibacterial Efficacies of Chlorhexidine Digluconate or Protamine Sulfate Combined with *Laminaria japonica* or *Rosmarinus officinalis* Extracts against *Streptococcus mutans*

MIN SEOK YOO¹, HYUNG-JOO JIN², AND SI YOUNG LEE¹*

¹Department of Oral Microbiology, College of Dentistry, Research Institute of Oral Science, Gangneung-Wonju National University, Gangneung, 210-702, Korea
²Department of Marine Molecular Biotechnology, College of Life Sciences, Gangneung-Wonju National University, Gangneung, 210-702, Korea

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Chlorhexidine digluconate inhibits oral bacteria and the formation of dental plaque. Protamine sulfate, a polycationic protein, exerts antibacterial activity by altering the cell wall of bacteria. Extracts of *Laminaria japonica* and *Rosmarinus officinalis* display antimicrobial effects against oral pathogens. The purpose of this study was to investigate the synergistic effect of chlorhexidine digluconate and protamine sulfate on the inhibitory activity of *L. japonica* and *R. officinalis* extracts against *Streptococcus mutans*, a major etiological agent for dental caries. Minimal inhibitory concentrations (MICs) of chlorhexidine digluconate, protamine sulfate, and *L. japonica* and *R. officinalis* extracts were determined by broth dilution method. Synergistic effect of chlorhexidine digluconate or protamine sulfate and extracts of *L. japonica* or *R. officinalis* was determined by fractional inhibitory concentration index (FIC). FIC demonstrated the synergistic effects of the different combinations of antibacterial agents. In this study, the use of sub-MIC of chlorhexidine digluconate or protamine sulfate with sub-MIC of *L. japonica* and *R. officinalis* extracts resulted in synergistic inhibitory effects of these antibacterial agents except for chlorhexidine digluconate and *L. japonica* combination.

Key words: Antibacterial agents / Combination / Synergy / Streptococci.

Chlorhexidine digluconate is a cationic biguanide microbicide with a broad spectrum of activities against bacteria and fungi, and is applied widely in both clinical and domestic concerns (Hiom et al., 1992). Chlorhexidine digluconate is known to express antimicrobial effects against a broad spectrum of oral pathogens (Addy and Langeroudi, 1984; Heling et al., 1992; Lang and Ramseier-Grossmann, 1981; Loe et al., 1976; Schiott et al., 1976; Steinberg et al., 1991). Protamine sulfate is an arginine-rich cationic polypeptide extracted from the sperm cells of vertebrates, such as salmon (Ando et al., 1973; Dixon and Smith, 1968). Protamine sulfate has been reported to display antimicrobial properties against a wide range of bacteria and yeasts (Kim et al., 2015). *Laminaria japonica* is a brown alga, which is consumed widely in Korea, Japan, and China. Ethanol extracts of *L. japonica* have been reported to display antimicrobial effects against oral pathogenic microorganisms, such as *Streptococcus mutans* (Kim et al., 2013a). *Rosmarinus officinalis* is an edible, fragrant, evergreen shrub containing active antimicrobial agents against pathogenic oral microorganisms (Kim et al., 2013b). Dental caries, the most common oral disease, is a multifactorial disease characterized by tooth destruction caused by interactions among bacteria within dental plaque, food, and saliva. *S. mutans* is believed to be the causative organism of dental caries, and is frequently isolated from human dental plaque (Loesche, 1986). In this study, we have examined the synergistic effect of chlorhexidine digluconate and protamine sulfate on the antibacterial activity of...
L. japonica and R. officinalis extracts against S. mutans.

Stock solutions of chlorhexidine digluconate (2.120 mg/ml) (Sigma-Aldrich Co., St. Louis, MO, USA) and protamine sulfate isolated from salmon (20 mg/ml) (Sigma-Aldrich Co.) were prepared in brain heart infusion broth (BHI; Becton, Dickinson and Company, Franklin Lakes, NJ, USA). The ethanol extracts of L. japonica and R. officinalis were prepared as described in previous studies (Kim et al., 2013a, b) to a final concentration of 100 mg/ml of ethanol. The stock solutions were filter-sterilized before use.

S. mutans ATCC 25175 was cultivated in BHI (Becton, Dickinson and Company) at 37ºC under aerobic conditions supplemented with 5% CO2 for 18 h.

The MICs were determined for the different antibacterials using a microdilution method in culture medium modified from that used in the standard antimicrobial susceptibility tests of the Clinical and Laboratory Standards Institute (CLSI). Using a microbial culture in the late log phase or stationary phase, a bacterial suspension equivalent to the 0.5 McFarland standard (approximately 1 × 10^8 CFU/ml) was prepared in each microbial culture medium. Serially diluted antimicrobial solutions were inoculated with bacteria in 96-well round-bottomed microtitration plates to a final concentration and a final volume of 5 × 10^5 CFU/ml and 100 µl. The microdilution trays were incubated at the same conditions as bacterial culture (described previously). The MIC was determined in samples incubated for 18 h. The microtitration plates were read visually, and the minimum concentration of extracts displaying no turbidity was recorded as the MIC. The experiments for determination of MIC were repeated at least thrice.

The determined MICs of chlorhexidine digluconate, protamine sulfate, and L. japonica and R. officinalis extracts against S. mutans are listed in Table 1. The MIC of chlorhexidine digluconate was 0.7 µg/ml and that of protamine sulfate was 78 µg/ml. The MICs of both L. japonica and R. officinalis extracts were 62.5 µg/ml against S. mutans.

The synergistic antibacterial effect of the chlorhexidine digluconate (or protamine sulfate) and L. japonica (or R. officinalis) combination was assessed by testing one of the agents at a constant concentration combined with dilutions of the other. The combined effect was calculated using the fractional inhibitory concentration (FIC) index (Odds, 2003). The FIC of an agent was obtained by dividing the MIC of the agent used in combination against the MIC of the agent used individually (FIC = MIC in combination/MIC individual). The FIC index was calculated as (MIC of chlorhexidine digluconate or protamine sulfate in combination/MIC of chlorhexidine digluconate or protamine sulfate individually) + (MIC of L. japonica or R. officinalis extract in combination/MIC of L. japonica or R. officinalis extracts used individually). A FIC index ≤ 0.5 indicated synergistic effect between the agents.

The FICs of chlorhexidine digluconate, protamine sulfate, and L. japonica and R. officinalis extracts against S. mutans are seen in Table 2. The FIC for the chlorhexidine digluconate (or protamine sulfate) + R. officinalis extract combination varied from 0.25 to 1 FIC, with an appreciable synergistic inhibitory effect on S. mutans. The FIC for protamine sulfate + L. japonica extract also varied from 0.253 to 1 FIC, with an appreciable synergistic inhibition of S. mutans. However, the FIC for chlorhexidine digluconate + L. japonica extract was within the range 0.503-1.
The antibacteria mechanisms of chlorhexidine digluconate and protamine sulfate were well reported, and the components for antibacterial activities of L. japonica and R. officinalis extracts were recently disclosed. Chlorhexidine digluconate binds to negatively charged sites on the bacterial cell wall via electrostatic interactions (Rolla et al., 1970; Rolla and Meisen, 1975), resulting in an impaired membrane that contributes to the leakage of intracellular organelles from the cytoplasm (Hidalgo and Dominguez, 2001; Ohta, 1990). The antimicrobial activity of protamine sulfate is suggested to be due to the electrostatic attraction between the positively charged protamine molecule and the negatively charged cell envelope, causing the inhibition or death of bacteria because of K⁺, ATP, and intracellular enzyme leakage (Johansen et al., 1997; Stumpe and Bakker, 1997). However, all of the possible antibacterial mechanisms of protamine have not yet been elucidated, and the mechanism by which protamine kills bacteria has been suggested to be different among various bacterial species (Johansen et al., 1996).

It has been reported recently that the depolymerized fucoidans form L. japonica showed antibacterial activity against Escherichia coli and Staphylococcus aureus (Liu et al., 2017). They showed that the bactericidal pathway of depolymerized fucoidans was through destruction of the cytomembranes. Cai et al. (2014) also reported that antimicrobial activity of L. japonica extract could be attributed to its ability to damage the cell wall and cell membrane. The inhibitory effect of rosemary was reported the result of the action of rosmarinic acid, rosmaridiphenol, carnosol, epirosmanol, carnosic acid, rosmanol and isorosmanol (Nieto et al., 2018). They interact with the cell membrane and produced the loss of membrane functionality and its structure (Nieto et al., 2018).

The synergistic in vitro activity of the chlorhexidine digluconate and protamine sulfate combination has been reported previously (Darouiche et al., 2008). Darouiche et al. (2008) had assessed the potential enhancing effect of protamine sulfate on the antimicrobial activity of chlorhexidine against planktonic catheter-associated organisms, and discovered either synergy or no interaction between chlorhexidine and protamine sulfate. The chlorhexidine digluconate-protamine sulfate combination was notably synergistic against E. coli, a common uropathogen.

The exact synergistic mechanism by which combinations of chlorhexidine digluconate, protamine sulfate, and L. japonica and R. officinalis extracts function remains unknown. However, it can be postulated that the exposure of bacteria to chlorhexidine digluconate and protamine sulfate increases cell wall permeability, allowing for the easy access of L. japonica and R. officinalis components to the bacterial cell membrane resulting damaging cytoplasmic membrane effectively. Because Cai et al. (2014) also showed that the components of L. japonica could inhibit the intracellular activities such as the glycolytic pathway (EMP), tricarboxylic acid (TCA) cycle, protein and nucleic acid synthesis, and DNA replication, it might be also possible that the damage of bacterial external structures by chlorhexidine digluconate and protamine sulfate facilitate the penetration of L. japonica extracts to the intracellular cytoplasm and accelerate the inhibition of the intracellular organelles. Our data which showed no synergistic effect in chlorhexidine digluconate and L. japonica combination need further studies for appropriate explanations.

In this study, we discovered that specific concentrations of chlorhexidine digluconate or protamine sulfate used in combination with L. japonica or R. officinalis extracts (also at specific concentrations) except for chlorhexidine digluconate and L. japonica combination showed an increase in the (synergistic) antibacterial effect against S. mutans, compared to their individual antibacterial activities. Chlorhexidine digluconate causes staining of the teeth and is known to have a bitter taste (Brown et al., 1986). The use of a combination of active antibacterial agents, which function in synergy, appears to be a logical pharmaceutical method to achieve the maximal therapeutic effect with minimal side effects (Shahriari et al., 2010).

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