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Antimicrobial Effects of Gatifloxacin on Canine Periodontopathic Bacteria *In Vitro*

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ABSTRACT

The aim of this study was to identify the potential antibacterial effects of gatifloxacin on one of canine periodontal pathogens, *Porphyromonas gulae*. The minimum inhibitory concentrations (MIC) of gatifloxacin and its bactericidal effects were investigated. Gatifloxacin inhibited the growth of the canine periodontopathic bacteria tested in broth. An MIC of 50 nM was found to be effective in inhibiting *P. gulae*. On performing adenosine triphosphate bioluminescence assay, gatifloxacin was found to exhibit bactericidal effects on the tested bacteria in a concentration-dependent manner. The safety of gatifloxacin in mammalian cells was evaluated by assessing the viability of rat bone marrow mesenchymal stem (BMMS) cells treated with gatifloxacin. Almost over 80% of BMMS cells survived after a 3-day culture when treated with 100 nM of gatifloxacin. These results indicate that locally administered gatifloxacin has the potential for being used to prevent canine periodontal infection.

1. INTRODUCTION

Dogs, the animals most closely related to humans, have been domesticated by humans for over 15,000 years, and recent research suggests that dogs have evolved via convergent evolution through the process of domestication [1]. Dogs are not just domesticated animals but are companions that live along with humans and belong to a unique species. It is no exaggeration to say that dogs are closely related to human life. Just like humans, dogs are inclined to develop periodontal disease within their lifespan. Approximately 80% of dogs over the age of 3 have periodontal disease [2]. The development of periodontal disease is closely related to the dog's diet as well as to the structure of the cuspids within the oral cavity. Periodontal

disease can develop through several causes such as neglecting daily tooth brushing, providing food that tends to leave residues in the dogs' mouths as well as the natural arrangement of their teeth. Dogs kept in a household are often fed "table scraps" by their owners via mouth-to-mouth means. As a result, the probability of dogs getting infected with human periodontal pathogenic bacteria increases, which is evidenced by the presence of human-derived *Porphyromonas gingivalis*, a major etiologic bacteria causing human periodontal diseases [3], in the canine oral cavity [4]. Periodontitis remains to be a serious infection in dogs as it is in humans. *P. gulae* can be detected in the oral cavity of dogs; this bacterium is a gram-negative obligate anaerobe, which is frequently detected in mammals other than humans. The 16S rRNA of *P. gulae* is homologous to that of *P. gingivalis*, which is detected in the human oral cavity, and shows 97% - 98% similarity [5]. *P. gingivalis* is negative for catalase activity, whereas *P. gulae* is positive [6]. Moreover, differences were observed between animal and human *Porphyromonas* species in terms of bacterial properties.

In our laboratory, potential risks for periodontal disease have been continuously researched. Large amounts of periodontopathic bacteria are reported to adhere to titanium surfaces, a metal normally used for dental implants [7]. In addition, the development of a defense system against bacterial adhesion on the surfaces of dental material was also a focus of our research [8]. Similar to periodontal disease in natural teeth, the presence of periodontopathic bacteria around titanium implants is a risk factor for peri-implantitis, which is defined as the inflammation that develops around an implant and is associated with bone loss, and a cause of implant failures [9-11]. The reduction of dental plaque accumulation or tartar adhesion to the tooth surface has been emphasized for the prevention of periodontal disease, such as peri-implantitis [12]. This also applies to canine periodontitis.

For developing a defense system to prevent peri-implantitis or periodontitis, we are currently conducting research on antibacterial activities of safe ingredients in foods derived from living organisms, such as the antimicrobial peptide protamine. Although protamine strongly inhibits the growth of *P. gingivalis*, its antimicrobial effects are transient, and sustainability of the medicinal benefit is difficult to achieve [13].

Subsequently, safer drugs that have fewer side effects were targeted. Our study in 2016 examined the inhibitory effects of gatifloxacin on the growth of periodontopathic bacteria [14]. Gatifloxacin, which chemical structure is shown in **Figure 1**, part of the 4th-generation fluoroquinolone drug family, has been frequently prescribed in the USA [15]. Although no serious side effects have been reported in dogs, the prescription of some fluoroquinolones has been discontinued or restricted due to the incidence of serious side effects, such as hypoglycemia and hepatotoxicity in humans, caused by systemic administration [16, 17]. In case of topical administration, gatifloxacin is mostly used as the primary drug for various eye infections [18].

Drug dosages administered per kg of body weight are often different for humans and dogs. When an antibacterial drug is administered to dogs, it is necessary to be mindful of this difference. This study aimed to investigate the antimicrobial effect of gatifloxacin on periodontal pathogens that infect canines as well as investigate the viability of rat cells in preventing periodontal infection by local administration.

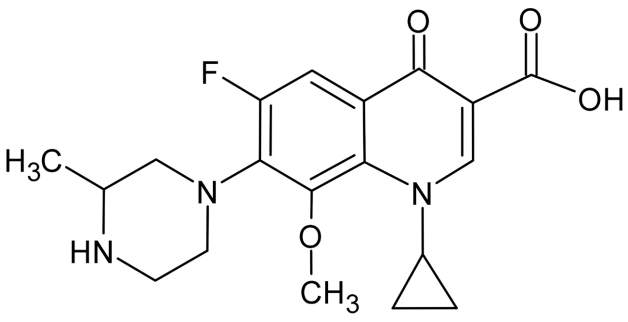

Figure 1. Chemical structure of gatifloxacin.

2. MATERIALS AND METHODS

2.1. Materials

Gatifloxacin was obtained from LKT Laboratories, Inc. (St. Paul, MN). For the cell proliferation assay, gatifloxacin was solubilized in Dulbecco's modified essential medium (Gibco, Grand Island, NY), which was then used as part of the culture medium described below. To evaluate minimum inhibitory concentration (MIC), gatifloxacin was solubilized in the broth as described below for the liquid culture of bacteria.

2.2. Bacteria and Culture Conditions

For plate culture, *P. gulae* ATCC 51700 was obtained from the American Type Culture Collection (Manassas, VA) and grown on plates containing tryptic soy agar (40 $g \cdot L^{-1}$; Becton, Dickinson and Company, Franklin Lakes, NJ) supplemented with 10% defibrinated horse blood (Japan Bio serum Inc., Tokyo, Japan), hemin (5 $g \cdot L^{-1}$; Sigma-Aldrich Corp., St. Louis, MO), and menadione (0.5 $g \cdot L^{-1}$; Sigma-Aldrich Corp.) [14]. Preculture was performed in an anaerobic chamber (N_2: 80%, H_2: 10%, CO_2: 10%) at 37˚C. For liquid culture, the bacteria were cultured in trypticase soy broth (30 $g \cdot L^{-1}$; Becton, Dickinson and Company) supplemented with 5 $g \cdot L^{-1}$ of hemin and 0.5 $g \cdot L^{-1}$ of menadione and incubated in an anaerobic chamber at 37˚C. The precultured colony was inoculated into a liquid broth and incubated for 2 - 4 days under the same conditions as those described for the plate culture.

2.3. Evaluation of Minimum Inhibitory Concentration

Broths containing gatifloxacin were used to determine MIC. Eleven serial concentrations of gatifloxacin were predetermined, ranging from 0 M to 1 μM for *P. gulae* cultures. The strain was inoculated into the broth and then incubated for 2 - 3 days in an anaerobic chamber at 37˚C. MIC was defined as the lowest concentration of gatifloxacin that would inhibit visible growth of bacteria after incubation. To confirm the reliability of the data, the experiments were performed four times.

2.4. Antibacterial Activity of Gatifloxacin against Canine Periodontopathic Bacteria

P. gulae strain was anaerobically grown at 37˚C until it reached the early stationary phase in the broth described above. The harvested cells were inoculated in the freshly prepared aseptic broth containing sufficient concentrations of gatifloxacin at 37˚C and examined for bacterial cell viability after 2 - 3 days. Bacterial cell viability was determined by adenosine triphosphate bioluminescence (ATP) assay using the BacTiter-Glo Microbial Cell Viability Assay kit (Promega, Madison, USA). A volume of BacTiter-Glo reagent equal to the volume of each bacterial suspension was added and briefly mixed. The luminescence of the solution was then recorded using the Gene Light Model GL-210A luminometer (Microtec Co., Ltd., Funabashi, Japan). The obtained value was expressed as a ratio to that at the start of incubation. The results were expressed as the mean ± standard deviation of the three experiments.

2.5. Mammalian Cell Viability Assay

Rat BMMS cells (Sigma-Aldrich, Tokyo, Japan) were used in this study. The culture medium consisted of a modification of Minimum Essential Medium (MEM *α*) (Thermo Fisher Scientific, Waltham, MA) supplemented with 10% fetal bovine serum (FBS French origin; Biowest, Nuaillé, France) and antibiotics. Cells subcultured at 37˚C in a humidified atmosphere with 5% CO_2 were suspended in MEM *α* at a concentration of 5.0×10^4 cells per well and then incubated for 24 h at 37˚C in a humidified atmosphere with 5% CO_2. The medium was replaced with a medium containing gatifloxacin at concentrations adjusted by stepwise dilution. The culture medium was refreshed every 2 days. After the completion of culture, cell viability was determined using a Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) for 3 days. Briefly, a 10-μL aliquot of CCK-8 solution was added to each well. The cells were then incubated for 90 min. Absorbance was measured at 450 nm using a microplate reader (iMark Microplate

Reader, Bio-Rad, CA, USA). The experiments were performed in triplicate. The data were expressed as survival rates.

2.6. Statistical Analysis

Statistical analyses were performed using SPSS Statistics version 25 (SPSS Inc., Chicago, USA). The data were analyzed using one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test, and probability (p) values < 0.05 were considered statistically significant.

3. RESULTS AND DISCUSSION

3.1. MIC Value of Gatifloxacin for Canine Periodontopathic Bacteria

As shown in Table 1, gatifloxacin showed a significant inhibitory effect on the growth of the canine periodontopathic bacteria strain tested. The MIC for *P. gulae* was 50 nM. These findings suggest that gatifloxacin exhibits selective inhibitory action against the growth of canine periodontopathic bacteria. The determination of MIC value of this drug is based on the turbidity of the bacterial fluid, although it is rather ambiguous to distinguish between viable or dead cells. In this case, statistical discussions are not appropriate. When our findings were compared to those of a recent study on the inhibitory effects of the drug against human periodontopathic bacteria *P. gingivalis* [14], which is genetically related to *P. gulae*, it was revealed that the MIC obtained in both studies were similar.

3.2. Inhibitory Effect of Gatifloxacin against *Porphyromonas gulae*

To further investigate the potential of gatifloxacin to inhibit the growth of periodontopathic bacteria, its bactericidal activity was assessed. As shown in Figure 2, gatifloxacin showed bactericidal activity in a concentration-dependent manner against the bacteria tested. The apparent growth from 0.25 nM to 5 nM was due to the reaction of the fluoroquinolone with the reagent, and the turbidity, shown in Table 1, was the same as that of the control (0 nM). Within this concentration range, the fluoroquinolone drug was considered to have little bactericidal effect. On using ANOVA and Tukey's multiple comparison test, a highly significant difference was observed between cultures containing high drug concentrations and the control culture without the drug in terms of inhibitory action. The number of viable bacterial cells decreased significantly as the bacteria were exposed to gatifloxacin concentrations of >100 nM ($p < 0.01$). This data supports the possibility that exposure to gatifloxacin suppresses bacterial growth during incubation, indicating that the drug may demonstrate an inhibitory effect on the growth of canine periodontopathogen *P. gulae*. This drug is reported to exert its effect by inhibiting bacterial DNA gyrase and topoisomerase IV [19, 20]. Against the bacteria tested in this paper, this drug is considered to have an adverse effect on a growth by a similar mechanism.

For a chronic canine periodontitis, a current professional dental treatment requires general anesthesia and surgical scaling [21, 22]. The results in this paper support that the local administration of gatifloxacin is effective for a nonsurgical therapy.

Table 1. Effect of gatifloxacin on the growth of periodontopathic bacteria *P. gulae* ATCC 51700 and minimum inhibitory concentration (MIC) of the reagent. Each mathematical symbol indicates the state of bacterial growth. ++: well grown, +: little growth, and −: no bacterial suspension was seen.

Bacterial stain	0 M	0.25 nM	0.75 nM	1.25 nM	2.5 nM	5 nM	50 nM	100 nM	250 nM	500 nM	750 nM	1 μM	MIC
P. gulae ATCC51700	++	++	++	++	++	++	+	−	−	−	−	−	50 nM

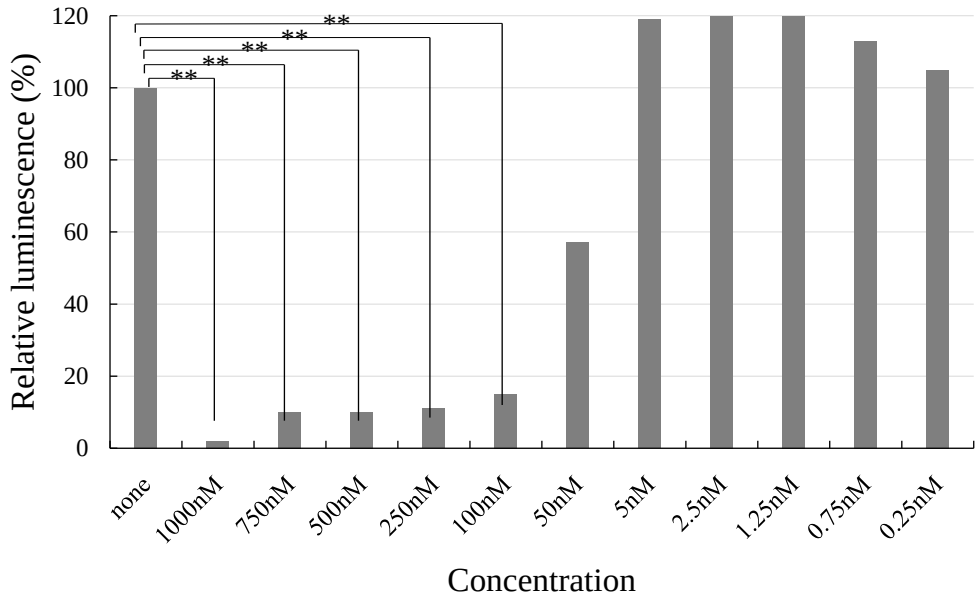

Figure 2. Influence of gatifloxacin on cell viability of periodontopathogen *P. gulae* ATCC 51700. Bars show the ratio of viable cell numbers after a 2-day incubation to numbers of initial cells at 0 M of gatifloxacin. Standard deviation is expressed by bar. Asterisks (**) indicate a value of $p < 0.01$, which was considered to be statistically significant.

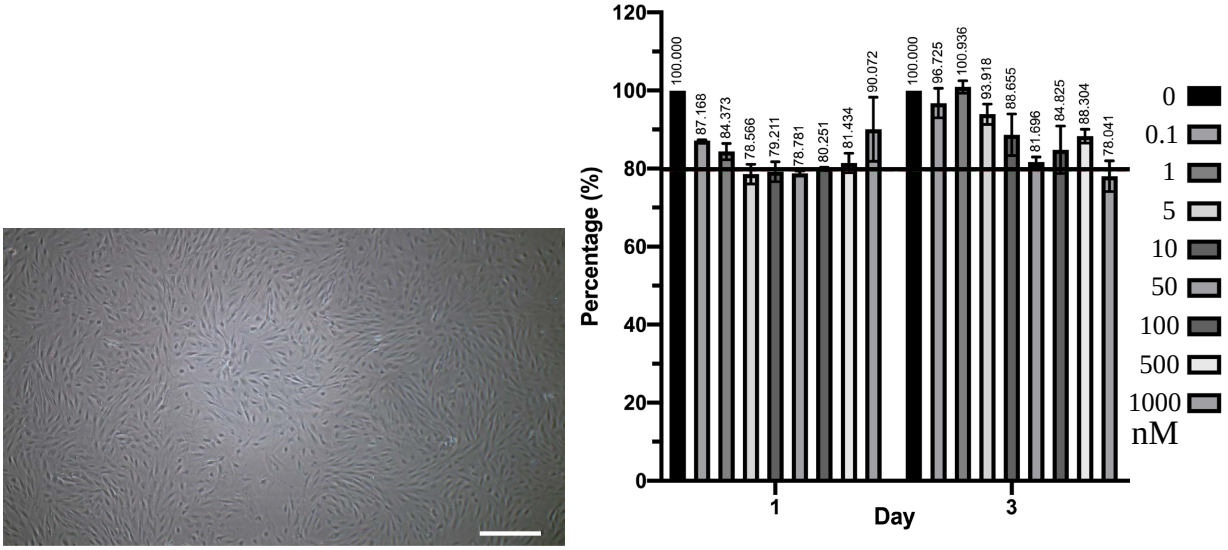

Figure 3. Survival rate of rat BMMS cells after 1-day and 3-day incubations in controls (0 M), which was defined as 100%, shown at each concentration of gatifloxacin. (a) Microscopic images of rat BMMS cells incubated with gatifloxacin-containing medium for 3 days. Scale bars represent 50 μm. (b) Growth of rat BMMS cells after a 1-day culture. (c) Growth of rat BMMS cells after a 3-day culture.

3.3. Cell Viability Assay in Mammalian Cells

As shown in **Figure 3(a)**, gatifloxacin had no morphological effect on the proliferation of mammalian

BMMS cells after a 3-day culture. Almost all seeded BMMS cells survived after a 1-day culture (**Figure 3(b)**), and 84.8% of the cells survived after a 3-day culture at 100 nM (**Figure 3(c)**), which is a concentration higher than the MIC of the bacteria tested. The fact that gatifloxacin is safe for mammalian cells after a 1-day culture suggests that it can be applied to other animals besides dogs. In studies of both Pneumococcal and Haemophilus strains, it is considered to be a drug that can clinically prevent the emergence of drug resistant bacteria (unpublished data from research of Kyorin Pharmaceutical Co. Ltd.) [19]. It is presumed that this reason is due to dual inhibition of both DNA gyrase and topoisomerase IV. The result of cell viability assay of gatifloxacin in rat cells was also strongly supported from the toxicity assay using normal dermal fibroblast cells derived from human adult connective tissue [14].

4. CONCLUSION

The results of the antibacterial activity and cell viability assays suggested that gatifloxacin exhibited bactericidal effects on *P. gulae* at low concentrations; however, it did not damage mammalian cells. The drug also exhibited antibacterial effects on *P. gulae* at concentrations above 100 nM, and most of the mammalian cells survived after a 3-day culture. This suggests that this drug would be harmless at an MIC, especially under conditions of short-term local administration. The results of this study suggest that gatifloxacin had an inhibitory effect on canine periodontal pathogens. Future studies need to test the efficiency of gatifloxacin against other pathogens that cause canine periodontal disease. In addition, gatifloxacin should also be tested against canine symbiotic microbiota. Nonetheless, further investigation is necessary to elucidate the properties of this locally administered drug and test the sustained release complexes established at our laboratory [23, 24]. One of our objectives is to develop a defense system against peri-implantitis and apply dental material technology to veterinary medicine to make contributions toward the treatment of severe periodontal disease in pets, such as dogs. The use of gatifloxacin for the treatment of canine periodontal diseases is expected to offer an immense benefit, leading to advancements in canine oral care.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest regarding the publication of this paper.

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