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In vitro Antimicrobial Effects of Silver Nanoparticles on Microorganisms Isolated from Dog with Otitis External

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Abstract : Silver nanoparticles have marked antimicrobial effects on several pathogens and have been used to control bacterial growth in humans. In the present study, we evaluated the antimicrobial efficacy of silver nanoparticles against the common causative pathogens of canine otitis external through counting of colony forming units. Silver nanoparticles showed significant dose-dependent antimicrobial effects on pathogens. In addition, we conducted antimicrobial susceptibility tests and compared the antimicrobial efficacy of silver nanoparticles. Microorganisms with a high resistance to antibiotics were also resistant silver nanoparticle with low concentration (5 μg/mL). However, in high concentration (15 μg/mL), almost 100% reduction in the number of CFUs of these pathogens was observed.

***Key words* :** antimicrobial effects, dog, otitis external, silver nanoparticles.

Introduction

Since ancient times, silver ions have been known to possess a strong antimicrobial effect, but their application in medicine has declined since the development of antibiotics. However, over the past few decades, nanotechnology has helped develop inorganic nanoparticles that have useful physical, chemical, and biological properties (2). These nanoparticles have emerged as antimicrobial agents owing to their extremely large ratio of surface area to volume (1,4,11,12). Among several metallic nanoparticles, silver nanoparticles in the size range of 10-100 nm have shown the highest antimicrobial effectiveness against several microorganisms, including multi-drug-resistant strains of bacteria (1,4,6,8,12).

In human clinics, the antimicrobial efficacy of silver nanoparticles has been demonstrated and silver ions have been used to control bacterial growth in several clinical applications (3,9,14,16,18) However, few studies have investigated the effectiveness of silver ions against common veterinary pathogens.

Otitis externa (OE) is one of the most commonly encountered diseases in dogs and microbial infections are the predominant causative and perpetuating factors of the disease. To treat OE, many clinicians have used antibiotic drugs empirically. However, the continued emergence of antimicrobial resistance is a primary concern and investigations into alternative methods for the treatment of infections are being performed worldwide.

In this study, we demonstrate the antimicrobial efficacy of silver nanoparticles against common causative pathogens of canine OE. For this research, we isolated the microorganisms from dogs with OE and compared the antimicrobial efficacy of silver nanoparticles with that of several commonly used antibiotics.

Materials and Methods

Silver nanoparticle formulation

The silver nanoparticles (Ag-NPs) powder used in this study were obtained from Sigma-Aldrich (No. 576832, Sigma-Aldrich, St. Louis, USA). The Ag-NPs were less than 100 nm in diameter and were purchased in a powder form. A solution of the Ag-NPs was prepared in distilled water and subsequent dilutions were made using tryptic soy broth (Becton Dickinson, Franklin Lakes, NJ, USA).

Bacterial strains

Fifteen dogs from different breeds that were affected with chronic OE were included as the subjects of this study. They were diagnosed with OE based on a clinical examination. The secretions inside the external auditory meatus were collected by rubbing with a sterilized cotton swab. Samples were cultured on a 5% sheep blood agar plate (Asan Pharmaceutical, Seoul, Korea) and MacConkey agar plate (Difco, Franklin Lakes, NJ, USA) and incubated aerobically for 24 h at 37°C to obtain single colonies. The microorganisms were identified by polymerase chain reaction using DNA extracted from the samples.

Assaying the antibacterial effects of Ag-NPs

The minimum inhibitory concentration of the Ag-NPs against microbial growth was determined by the plate counting method. The Ag-NPs powder was sterilized by irradiation with ultraviolet light for 1 h. The final concentrations of the Ag-NPs were 0, 5, 10, and 15 μg/mL and the bacterial cell concentration was adjusted to 10^5 colony-forming units (CFU)/mL using tryptic soy broth. Each culture was incubated at

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37ºC for 24 h with shaking at 100 rpm. Then, the cultured media (10 μL) was spread onto tryptic soy agar (Becton Dickinson) and incubated at 37ºC for 24 h. After incubation, the number of colonies growing on the agar was counted. The antibacterial effects of the Ag-NPs were estimated as the percentage reduction in the bacterial count, which was calculated using the following formula: Reduction (%) of bacteria = (B – A) / B × 100% (A: CFU counts with a given treatment, B: CFU counts with no treatment). Each isolate was experimentally analyzed by this method twice.

Antimicrobial susceptibility tests

Antimicrobial susceptibility was evaluated by the disc diffusion test using antimicrobial discs (Oxoid, Basingstoke, UK) for 12 antibiotics, namely amikacin (30 μg), amoxicillin-clavulanic acid (20/10 μg), cefixime (5 μg), cephalexin (30 μg), cephazolin (30 μg), cefradine (30 μg), ciprofloxacin (5 μg), enrofloxacin (5 μg), gentamycin (10 μg), levofloxacin (5 μg), trimethoprim/sulfamethoxazole (1.25/23.75 μg), and vancomycin (30 μg). The results were interpreted according to the guidelines of the Clinical and Laboratory Standards Institute (17).

Statistical analysis

The data from duplicate experiments are presented as the mean ± standard deviation. A paired *t*-test was performed for each treatment group, using SigmaPlot for Windows version 12.0 (Systat Software, San Jose, CA, USA), with comparison to the silver-free control plate. *P*-values < 0.05 were considered to indicate statistically significant differences.

Results

Bacterial strains

The prevalence and distribution of microorganisms isolated from the dogs with chronic OE are shown in Table 1. In total, 19 colonies were isolated and six bacterial species were identified from 15 samples. More than two species of bacteria were cultured from each of three samples.

Table 1. Bacterial isolation and identification results

Species	Number (%)
Staphylococcus pseudintermedius	10 (53)
Staphylococcus schleiferi	3 (15)
Pseudomonas aeruginosa	2 (10)
Enterococcus faecalis	2 (10)
Proteus mirabilis	1 (6)
Klebsiella pneumoniae	1 (6)
Total	19 (100)

Antibacterial effects of Ag-NPs

The antibacterial efficacy of the Ag-NPs was determined by analyzing the percentage reduction of bacterial populations that they caused. The results are shown in Table 2 and Fig 1. For all isolates, the Ag-NPs reduced the number of bacterial colonies by 80.90%, 95.37%, and 99.50% at 5, 10, and 15 μg/mL Ag-NPs, respectively. Among the 19 isolates, four isolates showed a growth inhibition of 100% when they were treated with 5 μg/mL Ag-NPs.

Antimicrobial susceptibility tests

The susceptibilities of six species to 12 antibiotics were examined (Table 3). Among 10 isolates of *Staphylococcus pseudintermedius*, one isolate showed resistance to all antibiotic drugs and the other two isolates showed resistance to 11 antibiotics. All the isolates of *Staphylococcus schleiferi* showed susceptibility to all 12 antibiotics.

Discussion

In the present study, we isolated microorganisms from dogs with OE and evaluated the antibacterial efficacy of silver nanoparticles against those isolates. In addition, we also evaluated the antimicrobial drug susceptibilities of the isolates. We studied 20 isolates from 15 dogs, and the distribution of

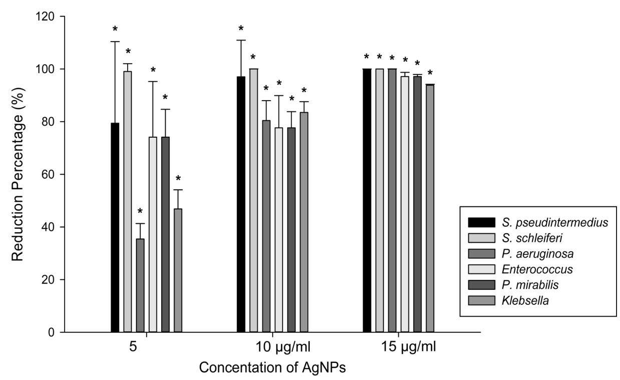

Fig 1. Percentage reduction in the number of colony-forming units for each isolated bacterial species in the presence of silver nanoparticles.
The data are displayed as the mean ± standard deviation. *P < 0.05.

Table 2. Antibacterial effects of silver nanoparticles at several concentrations. The values are shown as the mean ± standard deviation

	5 μg/mL	10 μg/mL	15 μg/mL
Staphylococcus pseudintermedius	79.42 ± 30.97	97.06 ± 13.89	99.94 ± 0.06
Staphylococcus schleiferi	99.04 ± 2.98	99.99 ± 0.03	100 ± 0.00
Pseudomonas aeruginosa	35.42 ± 2.95	80.43 ± 3.79	99.97 ± 0.06
Enterococcus faecalis	86.50 ± 10.56	77.67 ± 6.13	97.16 ± 0.79
Proteus mirabilis	74.13 ± 10.56	77.67 ± 6.13	97.16 ± 0.79
Klebsiella pneumoniae	46.83 ± 7.31	83.51 ± 4.08	93.85 ± 0.36

Table 3. Antibiotic susceptibilities of bacterial species isolated from dogs with chronic otitis externa

	Staphylococcus pseudintermedius (*n* = 10)	*Staphylococcus Schleiferi* (*n* = 3)	*Pseudomonas aeruginosa* (*n* = 2)	*Enterococcus faecalis* (*n* = 2)	*Proteus mirabilis* (*n* = 1)	*Klebsiella pneumoniae* (*n* = 1)
Cephalexin	7	3	0	0	1	1
Cefixime	4	2	0	0	1	1
Levofloxacin	4	3	2	2	1	0
STX	1	3	0	2	0	0
Ciprofloxacin	5	3	2	2	1	0
AMC	7	3	0	2	1	1
Cephazolin	7	3	0	0	0	0
Cefradine	6	3	0	0	0	0
Enrofloxacin	5	3	2	2	0	0
Gentamycin	4	3	2	0	0	0
Amikacin	9	3	1	0	1	1
Vancomycin	7	3	0	2	0	0

STX; trimethoprim/sulfamethoxazole, AMC; amoxicillin-clavulanic acid.

the microorganisms was similar to that reported in previous studies. *Staphylococcus* spp. were the pathogens most commonly isolated from ears with OE. The outcomes of the antimicrobial susceptibility tests were also similar to those in previous studies that evaluated the recent trends of antimicrobial drug resistance in Korean veterinary clinics (7,10).

The antibacterial activities of Ag-NPs against several pathogens were measured by counting CFUs on agar plates. After 24 h of incubation with several concentrations of Ag-NPs, there was a dose-dependent reduction in the number of CFUs of all the bacterial isolates. However, the antimicrobial potency of Ag-NPs differed among species. As in the antibacterial susceptibility tests, the antimicrobial potency of Ag-NPs at 5 μg/mL was lowest against *Pseudomonas aeruginosa,* while it was highest against *S. schleiferi*. However, the difference in potency was small, since there was a reduction of almost 100% in the number of CFUs for *P. aeruginosa*. For *S. pseudintermedius*, two isolates showed resistance to 11 of 12 antibiotics. Ag-NPs also had a less potent antimicrobial effect against the isolates showing broad drug resistance than against other isolates of *S. pseudintermedius*. On the basis of these results, we supposed that microorganisms show similar levels of resistance to antibiotics and Ag-NPs. However, as the concentration of Ag-NPs increased, the inhibition rate also increased. Thus, even for pathogens showing multi-drug resistance, Ag-NPs are a good choice as an alternative treatment for infections. Already, several previous studies have demonstrated that Ag-NPs have a strong antimicrobial efficacy, including effectiveness against multi-drug-resistant bacteria, and have suggested that they can form the basis for simple and cost-effective new types of bactericidal materials (8,12,15).

The most convenient approach for the treatment of canine OE is the topical application of a suitable medication. Baytril® Otic is a commonly used ear cleaning solution that contains enrofloxacin and silver sulfadiazine. These two ingredients are effective against bacterial and yeast infections of the ear and function by inhibiting DNA synthesis (13). However, Ag-NPs have more powerful antibiotic and anti-inflammatory effects (5,16). In a previous study, nanosilver-coated dressings, silver sulfadiazine dressings, and antibiotic dressings were applied to animal models of thermal skin injury, and the results demonstrated that the wound-healing properties of Ag-NPs were the greatest among the tested treatments (16). Another study investigated the application of Ag-NPs for topical use and found that the antibacterial spectrum of Ag-NPs was comparable to that of silver sulfadiazine, albeit at a 30-fold lower concentration (5). They also found that Ag-NPs interacted synergistically or additively with certain commonly used antibiotics. Thus, the combination of Ag-NPs and appropriate antibiotics can be expected to have a stronger antimicrobial efficacy than the commercial antibiotic formulations that are currently available for the treatment of canine OE.

In this study, Ag-NPs showed significant dose-dependent antimicrobial effects against several pathogens isolated from the ears of dogs with chronic OE. Since *in vivo* and *in vitro* conditions are quite different, more investigation is needed to clarify the clinical effectiveness and side effects of Ag-NPs before administering them to dogs. However, the findings of the present study suggest that Ag-NPs may have valuable applications as an alternative treatment for canine OE.

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