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The use of silver-coated ceramic beads for sterilization of *Sphingomonas* sp. in drinking mineral water

Dong-Wook Han[1], Min Sub Lee[1], Mi Hee Lee[2], Masakazu Uzawa[3] and Jong-Chul Park[1,2,]*
[1]*Department of Medical Engineering, Yonsei University College of Medicine, 134 Shinchon-dong, Seodaemun-gu, Seoul 120-752, Korea*
[2]*Brain Korea 21 Project for Medical Science, Yonsei University College of Medicine, 134 Shinchon-dong, Seodaemun-gu, Seoul 120-752, Korea*
[3]*Applied Science Co., Ltd., Ichikawa-si, Chiba 272-0822, Japan*
**Author for correspondence: Tel.: +82-2-361-5407, Fax: +82-2-363-9923, E-mail: parkjc@yumc.yonsei.ac.kr*

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Summary

It has been established that certain heavy metals including silver have antibacterial properties, and that they may be useful components of antibacterial materials. This knowledge of the antibacterial activity of silver has already been put to use to decrease the occurrence of urinary catheter infections and to develop topical antibacterial agents for burn victims. In this study, the ability of silver-coated ceramic beads (Ag-CBs), sintered at 375, 400, 425, or 450 °C, to sterilize *Sphingomonas* sp. in commercial mineral water was evaluated. Over 3% (w/v) Ag-CBs in mineral water completely sterilized *Sphingomonas* sp. after 6 h, regardless of the sintering temperature. The Ag-CBs sintered at 400 °C were the most useful since they did not produce clouding in the water and the bacteria were effectively killed. The antimicrobial activity of the 400 °C-sintered Ag-CBs was comparable to that of free silver ions at 15 μg/l, showing that the silver ions released from the Ag-CBs resulted in the complete sterilization of the microbial cells in mineral water. These results suggest that Ag-CBs present an easy-to-use option for mineral water sterilization.

Introduction

The antimicrobial activity of silver and of its ions, and those of copper and other metal ions, has been well known for centuries (Russell & Hugo 1994; Keleher *et al.* 2002; Nishioka *et al.* 2003). Of the metal ions, silver exhibits the highest toxicity for microorganisms and least toxicity to animal cells. The antimicrobial spectrum of silver is extensive, including gram-negative enterobacteria and gram-positive cocci (Zhao & Stevens 1998). Moreover, silver has been reported to be virucidal against poliovirus, adenovirus, bovine rotavirus, herpes, and vaccina virus and to have anti-algal properties (Shimizu *et al.* 1976; Abad *et al.* 1994; Scragg & Bonnett 2002).

During the last decade, water pollution has become a more pressing issue, and public concern continues to increase about the quality of natural mineral water. In most natural mineral waters, bacterial cells are assumed to exist under starvation conditions, and thus their growth is limited due to the low concentrations of suitable carbon and energy sources. Therefore, it is presumed that the conditions in most commercial mineral waters make it difficult for bacteria to grow (Taylor *et al.* 1983; Manaia *et al.* 1990). However, it has been reported that yellow-pigmented bacterial colonies are frequently encountered on nutrient agar isolation plates prepared from natural mineral water samples (Lee *et al.* 2001), and some bacteria from natural mineral waters have been isolated and characterized (Quevedo-Sarmiento *et al.* 1986; Manaia *et al.* 1990), but little information is available on yellow-pigmented bacteria and their sterilization.

This study was undertaken to investigate the antimicrobial activity of silver-coated ceramic beads (Ag-CBs), sintered at high temperatures (375, 400, 425, and 450 °C) against *Sphingomonas* sp. isolated from commercial mineral water, and to compare their effect with that of free silver ions.

Materials and methods

Bacterial strain isolation and culture conditions

The *Sphingomonas* sp. used in this study was originally isolated from contaminated commercial mineral water products and identified as *Sphingomonas* sp. by fatty acid composition analysis using gas chromatography performed by the Korean Culture Center of Microorganisms

(KCCM; Korean Federation of Culture Collections, Seoul, Korea).

An isolated colony was used to inoculate Tryptic Soy broth (Becton, Dickinson and Company, Sparks, MD, USA) and bacteria were grown to the stationary phase (18 h) at 25 °C in a rotary shaking water bath (100 rev/min). They were then sedimented by centrifugation at $3500 \times g$ for 10 min at 4 °C, and resuspended in sterile drinking water at a density of 1×10^5 colony-forming units (c.f.u.) per ml immediately prior to testing.

Preparation of silver-coated ceramic beads

Silver particles were coated onto the surfaces of ceramic beads (3 mm in diameter) by sintering at 375, 400, 425, or 450 °C in order to release silver ions (Ag^{2+}) in aqueous conditions. The surface roughnesses and thicknesses of Ag coatings on the prepared Ag-CB were observed under a laser microscope (Olympus Optical Co., Osaka, Japan).

Antimicrobial activity assay

After inoculating 50 ml of mineral water with *Sphingomonas* sp. (1×10^5 c.f.u./ml), the four different Ag-CB types were added to a final concentration of 1, 3, or 5% (w/v), and then incubated for 6, 24, 72, 144, or 312 h at room temperature. Considering that one Ag-CB weighs 0.25 ± 0.02 g, 1% (w/v) of Ag-CBs was achieved by adding approximately two beads (0.5 g) to each sample. At the end of incubation, the Ag-CB-exposed water was spread on plate count agar plates (Becton, Dickinson) in duplicate and cultured for 5 days at 25 °C in a humidified condition and the viable c.f.u./ml were then measured. The antimicrobial activity detection limit was determined as 0.01% of the relative c.f.u. (% of control).

For comparison purposes, free silver ions were added directly to 2.5×10^4 c.f.u./ml of bacteria-inoculated mineral water to a final concentration of 1 – 100 μg/l and then viable c.f.u.s were counted as described above. This test was designed to determine the amount of silver ions released from Ag-CB by comparing antimicrobial activities.

Results and discussion

Identification of the bacteria isolated from mineral waters

Yellow-pigmented bacteria were isolated from commercially available mineral water at several places in Tokyo, Japan. The isolates were yellow-pigmented, Gram-negative, aerobic and motile rods with a single polar flagellum. Furthermore, they were proven to contain ubiquinone 10 and 2-hydroxy-fatty acids without 3-hydroxy-fatty acids by gas chromatographic fatty acid composition analysis at KCCM. On the basis of these characteristics, the isolates were assigned to the genus *Sphingomonas*. The genus *Sphingomonas* was first proposed by Yabuuchi *et al.* (1990) and later amended by Takeuchi *et al.* (1993). Members of the genus *Sphingomonas* are yellow-pigmented, Gram-negative, aerobic, nonspore-forming, non-fermentative, motile or non-motile rods and are characterized chemotaxonomically by the presence of ubiquinone 10, 2-hydroxy-fatty acids and sphingolipids, and by the absence of 3-hydroxy-fatty acids (Wilkes *et al.* 1996; Lee *et al.* 2001).

Characteristics of the silver-coated ceramic beads

Four different types of Ag-CB were prepared at 375, 400, 425 or 450 °C. When immersed in mineral water, the Ag-CBs sintered at 375 °C caused precipitation in mineral water due to an abrupt release of silver ions, whereas those sintered at higher temperatures did not cause turbidity. This result indicated that silver ions not converted into silver metal remained excessively on the surfaces of ceramic beads sintered at 375 °C. Laser microscopic observations revealed greater roughnesses and thicknesses of Ag coatings on Ag-CB at higher sintering temperatures, suggesting that the conversion of silver ions into silver metal occurred more on the surfaces of the Ag-CBs sintered at 400 °C (Figure 1a) than on those sintered at 375 °C (Figure 1b).

Silver is perhaps the most common antimicrobial incorporated into solid materials, and it is one of the oldest antimicrobial agents on record. Silver ions are thought to inhibit bacterial enzymes, to interfere with electron transport, and to bind DNA (Feng *et al.* 2000). Moreover, they have been shown to protect catheter surfaces from *Pseudomonas aeruginosa* adhesion by a combination of lectins (Gu *et al.* 2001). Silver in the form of silver sulphadiazine is also one of the primary antimicrobials used to treat burn patients (Holder *et al.* 2003).

Antimicrobial activity of Ag-CB and free silver ions

As shown in Figure 2, the incubation of mineral water inoculated with *Sphingomonas* sp. in the presence of 1% (w/v) of Ag-CBs for 24 h resulted in complete sterilization, except for the Ag-CBs sintered at 450 °C (Figure 2a). On the other hand, over 3% (w/v) of Ag-CBs completely sterilized water after 6 h of exposure, irrespective of the sintering temperature (Figure 2b). In the absence of Ag-CBs, the number of viable c.f.u.s was about 1×10^5 c.f.u./ml. Conclusively, the exposure of bacteria-inoculated mineral water to more than 1% (w/v) of the Ag-CBs sintered at 400 °C resulted in complete sterilization after 6 h, and this antimicrobial activity was attributed to the continuous release of silver ions from the Ag-CB surface. When mineral water was inoculated with bacteria and then exposed to increasing concentrations of free silver ions, a marked decrease in c.f.u.s was also observed. Over 15 μg/l of silver ions reduced the initial inoculation density (2.5×10^4 c.f.u./ml) to lower than 100 c.f.u./ml (Figure 3). These results demonstrate that the antimicrobial activity of Ag-CB

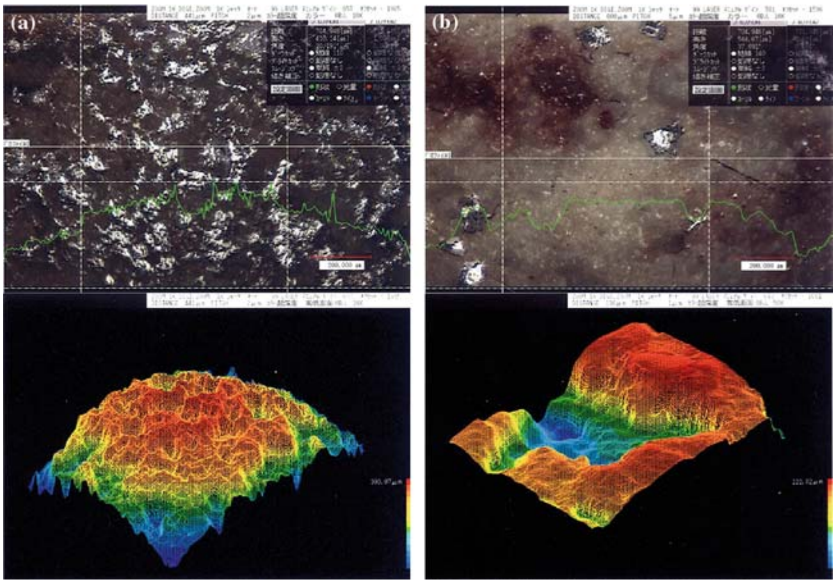

Figure 1. Laser microscopic observations of the surface of Ag-CBs sintered at 400 °C (a) and at 375 °C (b).

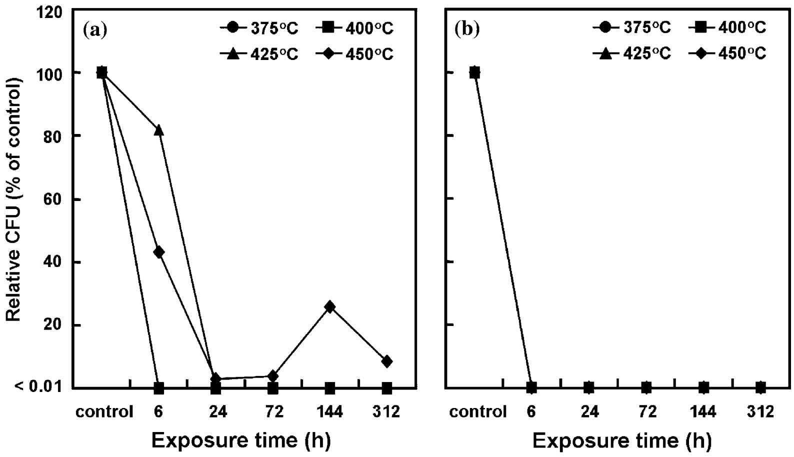

Figure 2. Relative c.f.u.s of *Sphingomonas* sp. in mineral waters exposed to 1% (w/v) Ag-CBs (a) and to over 3% (w/v) Ag-CBs (b) sintered at 375, 400, 425, or 450 °C.

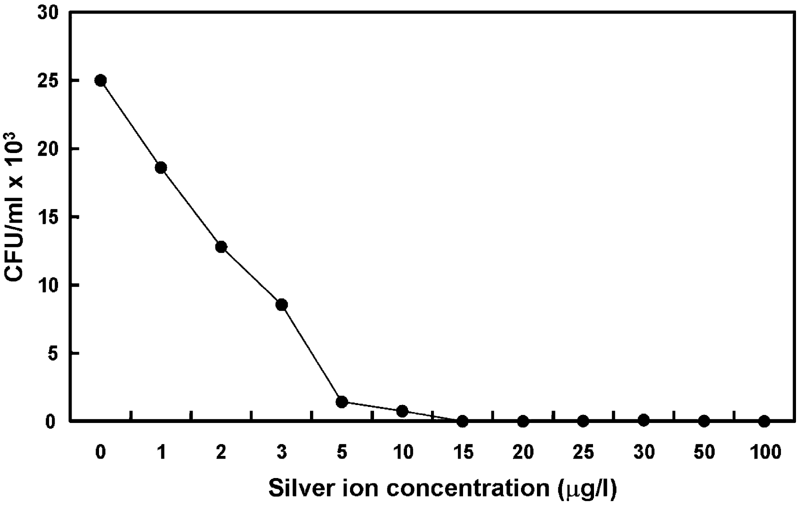

Figure 3. c.f.u.s of *Sphingomonas* sp. in mineral waters (2.5×10^4 c.f.u./ml) exposed to increasing concentrations (μg/l) of silver ions for 24 h at 25 °C in the dark.

sintered at 400 °C is comparable to that of free silver ions at 15 μg/l.

Silver's antimicrobial mode of action is multifaceted. Free, unbound Ag^{2+} ions disrupt transmembrane energy metabolism reversibly at low concentrations and irreversibly at high concentrations, the former effect is bacteriostatic and the latter bactericidal. The various mechanisms of action of silver depend on the concentration of free Ag^{2+} ions (Maillard 2002). Some of the suggested mechanisms are as follows:

1. Ag^{2+} forms insoluble compounds with sulfhydryl groups in the cell wall, thus deactivating enzymes involved in transmembrane energy generation and electrolyte transport (Liau *et al.* 1997).
2. Ag^{2+} blocks the bacterial respiratory chain in the cytochrome oxidase and NADH-succinate dehydrogenase region (Jansen *et al.* 1994).
3. Ag^{2+} enters the cell and makes the condensed form of DNA, which then loses its replicating ability (Feng *et al.* 2000).

Although silver ions are considered to have no detrimental effect on tissues, long-term silver toxicity is possible. If minute quantities of colloidal silver proteins or silver nitrate are ingested, they would accumulate over a long period and might cause argyria (White *et al.* 2003). Therefore, the Ag-CBs sintered at 400 °C would be safe enough to use as a mineral water sterilizer and might be the most effective because they did not cause turbidity in the water and the bacteria were completely sterilized. Before *in vivo* trials of silver-sterilized water, further study is required to determine the safe concentration of silver ions.

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