Comparison of Various Disinfectants on Bactericidal Activity Under Organic Matter Contaminated Environments

YOKO SATO1*, MASAYUKI ISHIHARA1, SHINGO NAKAMURA1, KOICHI FUKUDA1, MASAIRO KUWABARA2, TOMOHIRO TAKAYAMA2, SUMIYO HIRUMA1, KAORU MURAKAMI2, MASANORI FUJITA4, AND HIDETAKA YOKOE2

1Division of Biomedical Engineering, Research Institute, National Defense Medical College, 3-2 Namiki, Tokorozawa, Saitama 359-8513, Japan
2Department of Oral and Maxillofacial Surgery, National Defense Medical College, 3-2 Namiki, Tokorozawa, Saitama 359-8513, Japan
3Department of Plastic and Reconstructive Surgery, National Defense Medical College, Tokorozawa, Saitama 359-8513, Japan.
4Division of Environmental Medicine, Research Institute, National Defense Medical College, 3-2 Namiki, Tokorozawa, Saitama 359-8513, Japan.

Received 11 June, 2018/Accepted 2 October, 2018

The bactericidal activity of heated bio-shell calcium oxide (BiSCaO) powder suspension (pH 12.4), hypochlorous acid (HClO; pH 6), sodium hypochlorite (NaClO; pH 8), povidone-iodine (Isodine solution®), and chlorhexidine gluconate (Hibiscrub®) under organic matter contaminated environments were compared for tests conducted on wood scraps and pig skin pieces that were incubated with normal bacterial flora (total viable counts and coliform bacteria). The test results showed that BiSCaO suspension had higher bactericidal activity than HClO and NaClO. Furthermore, more than 10-fold higher concentrations of antiseptics such as povidone-iodine and chlorhexidine gluconate were required to achieve bactericidal activity comparable to that of BiSCaO suspension. Our results demonstrate the possibility of using BiSCaO suspension under organic matter contaminated environments as a disinfectant for environmental and food hygiene applications.

Key words: Hypochlorous acid / Sodium hypochlorite / Bio-shell calcium oxides / Bactericidal activity / Disinfectant.

Synthetic and naturally occurring antimicrobials are of interest owing to the availability of materials and effectiveness without undesirable side reactions. The purpose of this study is to compare and evaluate the bactericidal activity of several disinfectants on highly organic matter contaminated materials: heated bio-shell calcium oxide (BiSCaO) suspension, hypochlorous acid (HClO), sodium hypochlorite (NaClO), povidone-iodine, and chlorhexidine gluconate solutions.

Heated and pulverized scallop shell powder shows broad antimicrobial action against viruses, bacteria, spores, and fungi, and this material has been used in applications to prolong the shelf life of foodstuff as a food additive (Kubo et al., 2013). Scallop shells are a readily available resource with some portion used as food additives as well as in plastering and paving materials. However, most scallop shells become commercial waste, and shells piled on the shore in harvesting districts in Japan have caused serious problems, including offensive odors and soil pollution due to harmful materials contained in the shells, such as heavy metals (Sawai, 2011). A main component of scallop shells, calcium carbonate (CaCO₃), is converted to calcium oxide (CaO) when heated at over 1000°C.

Slurries of heated shell micro/nanoparticles (particle diameter range: 50 nm—10 µm) were prepared by grinding shells heated to over 1100°C in a wet bead-
grinding mill (Watanabe et al., 2014) and suspending the powder in sterile saline. The main component of this heated shell nanoparticle slurry was calcium hydroxide (Ca(OH)_2). Similarly, almost commercial heated shell powder products that are used as food additives are composed of Ca(OH)_2 instead of CaO. Bio shell calcium oxide (BiSCaO) powder (Bio Shell Co., Ltd.), which is also commercially available and used in this study, was prepared by heating at over 1100°C. The average diameter of the powder is about 7 µm, and its positive zeta-potential indicates that it is uniform and fine powder (data not shown).

Sanitation and decontamination in various fields such as healthcare and food processing are typically accomplished by broad-spectrum antibacterial and antiviral agents. Sodium hypochlorite (NaClO) solution (pH ≥ 8) is widely used for its activity as well as its low cost (Fukuzaki et al., 2006, Ardizzoni et al., 2009, Hao et al., 2013; Horiuchi et al., 2015). High concentrations (≥ 1000 ppm) are generally recommended in clinical settings for the inactivation of microorganisms in spilled body fluids, such as blood and feces due to the relatively poor antimicrobial activity of NaClO against some microorganisms such as Aspergillus oryzae, bacterial spores, poliovirus, and norovirus. However, at high concentration, the risk of producing carcinogens and poisonous substances such as trihalomethane in reactions with organic molecules increases. Another commonly used disinfectant, hypochlorous acid (HClO) solution (pH 6) is superior to NaClO solution (pH 8) in terms of having higher antimicrobial activity against a broad range of microorganisms and a broader spectrum even at low concentrations (Horiuchi et al., 2015). HClO carries lower risk of generating trihalomethane than NaClO, but HClO solution is less stable under various environmental conditions than NaClO and is thus easily inactivated. Notably, the presence of various organic compounds and inorganic ions results in the rapid depletion of HClO by oxidation reactions, a phenomenon that occurs more frequently for HClO than for NaClO (Ishihara et al., 2017).

A bath test sample (pH 6.9) with normal bacterial flora (total viable counts (TC) and coliform bacteria (CF)) was prepared by incubating leftover bath water with 10% Dulbecco’s Modified Eagle’s Medium (DMEM; Life Technologies Oriental, Tokyo, Japan) without fetal bovine serum and antibiotics at 37°C on shaker for 24 h.

To count the number of colony forming units (CFU), aliquots (1 mL of each mixture) were gently poured into individual dishes containing pre- aliquoted portions of simple and easy dry medium for TC or CF (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) (Sato et al. 2018), and the plates were incubated for 24 h in a 37°C incubator (Alp Co., Ltd., Tokyo, Japan). At the end of the incubation, the number of colonies in each dish reached an average CFU/mL for TC and CF of about 10 × 10^6 and about 2 × 10^6, respectively. To prepare wood scraps and pig skin pieces with normal bacterial flora, wood scraps (1.2 cm × 1.2 cm × 0.2 cm) and pig skin pieces (1.2 cm × 1.2 cm × 0.2 cm) were placed in bath test sample water with normal bacterial flora, incubated at 37°C on a shaker for 24 h, rinsed twice with pure water (Fukuda et al., 2017), put into 10 mL pure water, and vortexed hardly for 1 min. The average number of TC and CF in wood scraps was 3 × 10^6 and 2 × 10^6 CFU/mL, respectively. The average number of TC and CF in pig skin piece samples was 8 × 10^6 and 5 × 10^6 CFU/mL, respectively.

Disinfectant solutions were prepared as follows. BiSCaO powder (1.8 g) was added into 1 L of pure water and mixed well to prepare 1800 ppm BiSCaO suspension (pH 12.4) that was then diluted to three concentrations (600, 200, and 66 ppm). To prepare HClO and NaClO of approximately 1800, 600, 200, and 66 ppm, 0.5% NaClO (Yoshida Pharmaceutical Corp., Tokyo, Japan) was diluted into pure water, and the pH was adjusted to 6 and 8, respectively, with 1 N hydrochloric acid (HCl). The concentration of HClO and NaClO solutions was measured as residual chlorine (hypochlorous acid molecules (HClO) and hypochlorous ions (ClO^-)) using ClO-selective test papers (high concentration, 25–500 ppm; low concentration, 1–25 ppm; Kyoritu Check Laboratory Corp., Tokyo, Japan). Povidone-iodine and chlorhexidine gluconate solutions were prepared by diluting isodine solution (7%, Meiji Uga Co., Ltd., Tokyo, Japan) and Hibiscrub® (4%, Sumitomo Dainippon Pharma Co., Ltd., Tokyo, Japan) in pure water to achieve concentrations of 18,000, 6000, 2000, and 660 ppm. Pure water was used for the control.

First, we evaluated the antibacterial activity of each solution against the bath test sample (Figure 1). The experiments were repeated four times. Briefly, 10 mL of BiSCaO suspension (1800, 600, 200, and 66 ppm) was combined with 10 mL of bath test sample, mixed well (pH 11.8, 10.9, 9.5, and 8.2, respectively) and incubated at room temperature for 15 min. The effective concentration of each test solution was 900, 300, 100, and 33 ppm BiSCaO suspension. Similarly, NaClO and HClO solutions were added into the bath test sample. After mixing, the pH of HClO and NaClO treatments were adjusted to pH 6 and 8, respectively. The concentration of residual chlorine before mixing was 900, 300, 100, and 33.3 ppm of both HClO and NaClO solutions. At 15 min after mixing, the concentration of residual chlorine decreased to 450, 100, 10, and 1 ppm in HClO, and 600, 150, 30, and 5 ppm in NaClO, respectively. BiSCaO suspension and HClO solution (300 ppm...
BACTERICIDAL ACTIVITY OF SOME DISINFECTANTS

Wood scraps was showed as follow. After the first cleansing, it decreased to 500, 100, 20, and 2.5 ppm in HClO and 600, 150, 30, and 5 ppm in NaClO, respectively. After the third cleansing, it decreased to 700, 200, 50, and 15 ppm in HClO, and 800, 250, 75, and 25 ppm in NaClO, respectively. Similarly, the decrease of the concentration of residual chlorine after cleansing pig skin sample was showed as follow. After the first cleansing, it decreased to 400, 75, 10, and 1 ppm in HClO and 500, 100, 20, and 2.5 ppm in NaClO, respectively. After the third cleansing, it decreased to 600, 150, 25, and 5 ppm in HClO, and 700, 200, 50, and 10 ppm in NaClO, respectively. For the 900 ppm BiSCaO suspension, both TC and CF were completely reduced in the wood scraps, while only low levels of TC and CF were viable at 900 ppm of HClO and NaClO. The total removal of TC for pig skin piece samples couldn’t be achieved, even with 900 ppm BiSCaO suspension, however, its antibacterial activity was higher than that in HClO and NaClO at each concentration. On the other hand, the povidone-iodine and chlorhexidine gluconate, which are antiseptics used in clinical settings, required about 10-fold higher concentration to achieve disinfection activity comparable to that of BiSCaO suspension.

Next, we tested the antibacterial activity of each disinfectant solution against wood scraps and pig skin pieces. The experiments were repeated four times, respectively. To the wood scraps or pig skin pieces incubated with the bath test sample water, 10 mL of one of the disinfectant solutions was added, and the entire mixture was gently vortexed for 30 sec. The cleansing procedure with each fresh disinfectant solution was repeated three times before determining the CFU of TC or CF in each supernatant.

The concentration of residual chlorine before cleansing contaminated materials was 900, 300, 100, and 33.3 ppm of both HClO and NaClO solutions. The decrease of the concentration of residual chlorine after cleansing wood scraps was showed as follow. After the first cleansing, it decreased to 500, 100, 20, and 2.5 ppm in HClO and 600, 150, 30, and 5 ppm in NaClO, respectively. After the third cleansing, it decreased to 700, 200, 50, and 15 ppm in HClO, and 800, 250, 75, and 25 ppm in NaClO, respectively. Similarly, the decrease of the concentration of residual chlorine after cleansing pig skin sample was showed as follow. After the first cleansing, it decreased to 400, 75, 10, and 1 ppm in HClO and 500, 100, 20, and 2.5 ppm in NaClO, respectively. After the third cleansing, it decreased to 600, 150, 25, and 5 ppm in HClO, and 700, 200, 50, and 10 ppm in NaClO, respectively. For the 900 ppm BiSCaO suspension, both TC and CF were completely reduced in the wood scraps, while only low levels of TC and CF were viable at 900 ppm of HClO and NaClO. The total removal of TC for pig skin piece samples couldn’t be achieved, even with 900 ppm BiSCaO suspension, however, its antibacterial activity was higher than that in HClO and NaClO at each concentration. On the other hand, the povidone-iodine and chlorhexidine gluconate, with ten-fold higher concentration than BiSCaO suspension, could remove neither TC nor CF of wood scraps and pig skin pieces.
Regarding the possible side effect of the strong alkaline and the long-term safety effects in both humans and for the environment.

HClO solution at around pH 6, which is prepared by the addition of NaClO and HCl to sterile water or saline, has been demonstrated to have excellent in vitro bactericidal properties at lower concentrations (\(50 - 200\) ppm) than NaClO for gram positive bacteria such as S. aureus, B. cereus, and B. subtilis, as well as gram negative bacteria such as P. aeruginosa (Hakim et al., 2015; Ono et al., 2012; Hao et al., 2013; Sakarya et al., 2014). However, HClO readily reacts with various NH\(_2\)- or CHO-containing organic (e.g., protein, amino acid, and carbohydrate) and inorganic compounds, and it leads to the rapid consumption of HClO under organic matter contaminated environments (Ishihara et al., 2017, Sato et al., 2018). Some reports suggest that HClO can interact with primary amino-groups (\(-\text{NH}_2\)) in organic compounds such as amino acids, thereby generating chloramine groups (\(-\text{NH}_2\text{Cl}\) or \(-\text{NHCl}_2\)) that are known to show oxidizing properties and to exhibit antimicrobial activity (Gottardi et al., 2013; Thomass et al., 1986). In fact, residual chlorine levels of HClO in 5% DMEM, which includes glutamine, rapidly decreased with the rate that was apparently dependent on the

Although CaO hydration, which generates an alkaline condition, is considered to be the primary disinfection mechanism of BiSCaO, the disinfection activity of BiSCaO suspension for both TC and CF was much higher than that achieved by NaOH solution at the same pH (data not shown). Another possible mechanism for explaining the high disinfection activity of BiSCaO suspension is that the hydroxide ion (OH\(^-\)) concentration of the thin water layer formed around BiSCaO micro-particles might be higher than in equilibrant solution (Kubo et al. 2013). Further, heated shell powder has higher disinfection activity against biofilms of E. coli (Kubo et al., 2013) and Listeria sp. (Shimamura et al., 2015) in terms of deactivation and removal. When BiSCaO particles are surrounded by a thin aqueous layer of abundant OH\(^-\) groups in contact with the contaminated surface of wood or pig skin pieces, bacterial cells might be damaged and removed. It has also been proposed that active oxygen species generated from heated scallop-shell powder may also contribute to strong disinfection activity (Kubo et al., 2013; Shimamura et al., 2015). Additionally, calcium ions, which is the other product in hydration of CaO, is thought to make the bacteria’s living environmental worse (Jason W et al., 2009). However, further investigation is required regarding the possible side effect of the strong alkaline and the long-term safety effects in both humans and for the environment.

HClO solution at around pH 6, which is prepared by the addition of NaClO and HCl to sterile water or saline, has been demonstrated to have excellent in vitro bactericidal properties at lower concentrations (50 – 200 ppm) than NaClO for gram positive bacteria such as S. aureus, B. cereus, and B. subtilis, as well as gram negative bacteria such as P. aeruginosa (Hakim et al., 2015; Ono et al., 2012; Hao et al., 2013; Sakarya et al., 2014). However, HClO readily reacts with various NH\(_2\)- or CHO-containing organic (e.g., protein, amino acid, and carbohydrate) and inorganic compounds, and it leads to the rapid consumption of HClO under organic matter contaminated environments (Ishihara et al., 2017, Sato et al., 2018). Some reports suggest that HClO can interact with primary amino-groups (\(-\text{NH}_2\)) in organic compounds such as amino acids, thereby generating chloramine groups (\(-\text{NH}_2\text{Cl}\) or \(-\text{NHCl}_2\)) that are known to show oxidizing properties and to exhibit antimicrobial activity (Gottardi et al., 2013; Thomass et al., 1986). In fact, residual chlorine levels of HClO in 5% DMEM, which includes glutamine, rapidly decreased with the rate that was apparently dependent on the

**FIG. 2.** Antibacterial activity of disinfectants against wood scraps

The number of colony forming unit (CFU) of (A) total viable microbe counts (TC) and (B) coliform bacteria (CF) of contaminated wood scraps that were gently vortexed three times for 30 sec with bio-shell calcium oxides (BiSCaO) solution, sodium hypochlorite (NaClO) solution, and hypochlorous acid (HClO) suspension were measured (\(n = 4\)). Similarly, (C) TC and (D) CF for povidone-iodine and chlorhexidine gluconate solution vortexed three times for 30 sec were measured (\(n = 4\)). Pure water was the control.
that application of BiSCaO may be a safe and effective material for use as a disinfectant in environmental and food hygiene settings.

ACKNOWLEDGMENT

This study was partially supported by the Ministry of Education, Culture, Sports, Science and Technology of the Government of Japan (grant No. 17K19861). The authors would like to thank Forte Science Communication (https://www.forte-science.co.jp) for the English language review.

REFERENCES

Ardizzoni, A., Blasi, E., Rimoldi, C., Giardino, L., Ambu, E.,
Ishihara, M., Murakami, K., Nakamura, S., Sato, Y., Fukuda, K., Fujita, M., Kiyosawa, T., and Yokoe, H. (2017) Cleaning technique using high-velocity steam-air micromist jet spray. J. Med. Engineer. Technol. 41, 522-528.

Fukuzaki, S. (2006) Mechanisms of actions of sodium hypochlorite in cleaning and disinfection processes. Biocontrol Sciences, 11, 147-157.

Gottardi, W., Debabov, D., and Nagi, M. (2013) N-chloramine, a promising class of well-tolerated topical anti-infectives. Antimicrob. Agents Chemother. 57, 1107-1114.

Hakim, H., Thanmakarn, C., Suguro, A., Ishida, Y., Kawamura, A., Tamura, M., Satoh, K., Tsujimura, M., Hasegawa, T., and Takehara, K. (2015) Evaluation of sprayed hypochlorous acid solutions for their virucidal activity against avian influenza virus through in vitro experiments. J. Vet. Med. Sci., 77, 211-215.

Hao, X. X., Li, B. M., Zhang, Q., Lin, B. Z., Ge, L. P., Wang, C. Y., and Cao, W. (2013) Disinfection effectiveness of slightly acidic electrolyzed water in swine barns. J. Appl. Microbiol., 115, 703-710.

Horiuchi, I., Kawata, H., Nagao, T., Imaohji, H., Murakami, K., Kino, Y., Yamasaki, H., Koyama, A. H., Fujita, Y., Gota, H., and Kuwabara, T. (2015) Antimicrobial activity and stability of weakly acidified chlorous acid water. Biocontrol Sci., 20, 43-51.

Ishihara, M., Murakami, K., Fukuda, K., Nakamura, S., Kuwabara, M., Hattori, H., Fujita, M., Kiyosawa, T., and Yokoe, H. (2017) Stability of weakly acidic hypochlorous acid solution with microbiocidal activity. Biocontrol Sci., 22, 223-227.

Ishihara, M., Nguyen, V. Q., Mori, Y., Nakamura, S., and Hattori, H. (2015) Adsorption of silver nanoparticles onto different surface structures of chitin/chitosan and correlations with antimicrobial activities. Int. J. Mol. Sci., 16, 13973-13988.

Jason W., Jack S., Geli G., Yong-Dong W., and Elaine I. (2008) Calcium efflux is essential for bacterial survival in the eukaryotic host. Mol Microbiol. 70, 435-444.

Kubo, M., Ohshima, Y., Irie, F., Kikuchi, M., Sawai, J. (2013) Disinfection treatment of heated scallop-shell powder on biofilm of Escherichia coli ATCC 25922 surrogated for E. coli O157:H7. J. Biomater. Nanobiotechnol. 4, 10-19.

Ono, T., Yamashita, K., Murayama, T., and Sato T. (2012) Microbicidal activity of weak acid hypochlorous solution on various microorganisms. Biocontrol Sci., 17, 129-133.

Sakarya, S., Gunay, N., Karakulak, M., Ozturk, B., and Ertugrul, B. (2014) Hypochlorous acids: an ideal wound care agent with powerful microbicidal, antibiofilm, and wound healing potency. Wounds, 26, 342-350.

Sato, Y., Ishihara, M., Fukuda, K., Nakamura, S., Murakami, K., Fujita, M., and Yokoe, H. (2018) Behavior of nitrate nitrogen and nitrite nitrogen in drinking waters. Biocontrol Sci., 23, 139-143.

Sawai, J. (2011) Antimicrobial characteristics of heated scallop shell powder and its application. Biocontrol Sci., 16, 95-102.

Shimamura, N., Irie, F., Yamakawa, T., Kikuchi, M., and Sawai, J. (2015) Heated scallop-shell powder treatment for deactivation and removal of Listeria sp. biofilm formed at low temperature. Biocontrol Sci., 20, 153-157.

Thomas, E. L., Grisham, M. B., and Jefferson, M. M. (1986) Preparation and characterization of chioramines. Methods Enzymol., 132, 569-585.

Watanabe, T., Fujimoto, R., Sawai, J., Kikuchi, M., Yahata, S., and Satoh, S. (2014) Antibacterial characteristics of heated scallop-shell nano-particles. Biocontrol Sci., 19, 93-97.

Wiegand, C., Abel, M., Ruth, P., Elsner, P., Hipler, U-C. (2015) pH influence on antibacterial efficacy of common antiseptic substances. Skin Pharmacol. Physiol., 28, 147-158.