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Report

Microbicidal Effect of Sodium Chlorite in Combination with Caffeine at a Neutral pH

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Sodium chlorite ($NaClO_2$) is applied as a disinfectant for the sanitization of food and environmental surfaces. $HClO_2$ (p*K*a = 1.86) is believed to be the main species responsible for the antimicrobial effects, whereas $NaClO_2$ solution ($[HClO_2] / [ClO_2^-] = 10^{-5.64}$ at pH 7.5) shows only weak antimicrobial activity at neutral pH conditions. However, $NaClO_2$ solution at a neutral pH has the advantages of having very low reactivity to organic materials and high stability for long-term storage at around room temperature. In our previous screening of food additives, phytochemicals, and related compounds, we found that caffeine could strongly promote the antimicrobial effects of $NaClO_2$ solution at a neutral pH. Caffeine is a purine alkaloid found in nearly 100 plant species that has very weak antibacterial properties against many bacteria. In the present study, we evaluated the antimicrobial activity of $NaClO_2$ in combination with caffeine against *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Candida albicans* strains. We found that caffeine strongly potentiated the antimicrobial action of $NaClO_2$ in all four strains, indicating that this combination has potential as a disinfectant.

Key words sodium chlorite, disinfectant, caffeine, bactericide, fungicide

INTRODUCTION

The public health importance of preventing the spread of infectious diseases has been gaining increasing attention over the last several decades. Many emerging and re-emerging infectious diseases derived from microorganisms have been appearing, including methicillin-resistant *Staphylococcus aureus*-derived diseases, drug-resistant tuberculosis, cholera caused by *Vibrio cholerae*, and hemorrhagic colitis caused by *Escherichia coli* (EHEC).[1] Thus, there is a clear need for effective disinfectants for inactivating microorganisms without adversely affecting the human body.

Sodium hypochlorite (NaClO) has been widely used as a disinfectant for its high oxidizing power, but its disinfecting efficacy is markedly reduced by the presence of organic matter or biological substances, which is a serious disadvantage.[2-4] Another form of chlorine that is based on sodium chlorite ($NaClO_2$) has been applied for the sanitization of food and environmental surfaces. Acidification of $NaClO_2$ solution results in the protonation of the chlorite ion (ClO_2^-) to produce chlorous acid ($HClO_2$). Since $HClO_2$ is an acid with a p*K*a of 1.86,[5] the equilibrium between $HClO_2$ and ClO_2^- is dependent on the pH of the solution. $HClO_2$ is believed to be the main species responsible for the antimicrobial effects,[6,7] whereas $NaClO_2$ solution shows only weak antimicrobial activity at neutral pH conditions. However, $NaClO_2$ solution at a neutral pH has the advantages of having very low reactivity to organic materials and high stability for long-term storage at around room temperature.

The molecular concentration ratio of the acid and corresponding base of chlorous acid is given by the Henderson-Hasselbalch equation ($\log [HClO_2] / [ClO_2^-] = pK\text{a} - \text{pH}$).[2] As $\log [HClO_2] / [ClO_2^-] = -5.64$ at pH 7.5 (calculated with a p*K*a = 1.86), the relative number of $HClO_2$ and ClO_2^- molecules is $10^{-5.64}$, or about 1/440,000 at pH 7.5. In contrast, the antimicrobial chlorite $HClO_2$ is formed under strong acidic pH conditions, and is further converted rapidly to chlorine dioxide (ClO_2), which is considered to be a principle component of the strong antimicrobial activity.[4,8] Because ClO_2 is easily released into the air, the antimicrobial activity of acidic $NaClO_2$ solution decreases quickly after preparation. Previously, we performed a screen of food additives, phytochemicals, and related compounds to identify chemicals that could promote the antimicrobial effect of $NaClO_2$ solution at a neutral pH. We found that caffeine could strongly potentiate the antimicrobial action of $NaClO_2$ solution at pH 7. 5.

Caffeine (1,3,7-trimethylxanthine) is a purine alkaloid present in nearly 100 plant species.[9] The main natural sources of caffeine are tea (*Camellia sinensis* L.), coffee (*Coffea arabica* L.), cocoa (*Theobroma sinensis* L.), and mate (*Ilex para-*

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guariensis). The effects of caffeine on human health have been extensively studied.[10-12] In addition, some studies have reported that caffeine has very weak antibacterial properties against many bacteria and fungi, including *S. aureus*, *Pseudomonas aeruginosa*, *E. coli,* and *Candida albicans*.[13,14] In contrast, Hosseinzadech *et al*. have reported that caffeine (up to 8 mg/mL (41.2 mM)) did not show any antibacterial effects on *S. aureus* and *P. aeruginosa*.[15]

In this study, we evaluated the antibacterial and antifungal activities of $NaClO_2$ in combination with caffeine against *Acinetobacter baumannii*, *P. aeruginosa*, *S. aureus*, and *C. albicans*.

MATERIALS AND METHODS

Bacterial Strains The *P. aeruginosa* NBRC 13275, *A. baumannii* NBRC 109757, *S. aureus* NBRC 13276, and *C. albicans* NBRC 1594 strains were purchased from the National Institute of Technology and Evaluation (Tokyo, Japan).

Chemicals for Screening We screened for possible chemicals among "food additives, phytochemicals, and related compounds", as follows. Thirty-three tannins and related compounds contained in the laboratory's tannin-library as indicated in our reference.[16] The other 58 compounds are acesulfame potassium, adenine, adenosine, 5'-adenylate, adipic acid, 5-aminolevulinic acid, azoxystrobin, betaine, biotin, caffeic acid, **caffeine**, calcium phytate, choline chloride, cinnamic acid, citric acid, curcumin, 5'-cytidylate, cytidine, cytosine, disodium succinate, fludioxonil, glycerol, guanine, guanosine, 5'-guanylate, 1-hexadecanoyl pyridinium chloride, hinokitiol, 1-hydroxyethane-1,1-diphosphonic acid, L-hydroxyproline, imazalil, inosine, myo-inositol, malic acid, D-mannitol, methylsulfonium chloride, nicotinamide, pantothenate, 2-phenylphenol, potassium sorbate, pyridoxine hydrochloride, pyrimethanil, phytic acid, protamine sulphate, riboflavin, saccharin, sodium acetate, sodium benzoate, sodium carboxymethylcellulose, sodium lactate, sodium 5'-inosinate, sodium nitrate, sodium saccharin, D-sorbitol, tartaric acid, taurine, thjaplicin, trigonelline, and xylitol. These chemicals were prepared to neutral pH, if needed, after solubilizing those with distilled water. The sterilization of the chemicals was performed using 0.22 μm nitrocellulose filter.

Growth Conditions The stock bacterial cultures were maintained at -70°C with 30%W/V glycerol. The liquid culture broth was Luria Bertani medium (1% W/V polypeptone (Beckton Dickinson and Company, Franklin, NJ, USA), 0.5% W/V yeast extract (Beckton Dickinson and Company), and 0.5% W/V NaCl) containing 0.5% W/V D-glucose and 5 mM potassium phosphate buffer (pH 7.5) (LBG medium). Preculturing of the bacteria and fungus was carried out for 16 h. The amounts of sodium chlorite to add into each microbial culture medium were determined as a property of weak growth inhibition (approximately 10-40% of full growth inhibition) by the preliminary experiments. For the primary screening by use of 96-well microplate similar to above growth conditions, *Staphylococcus aureus* NBRC 13276 was cultured in the presence or absence of 100 μg/mL of $NaClO_2$ and several amounts of chemical compounds for the screening. We found that only caffeine could strongly potentiate the antimicrobial action of $NaClO_2$ solution at pH 7. 5. For the practical experiments, the cultures were inoculated at 1/50 into the test culture media containing or not containing (control) sodium chlorite (100 μg/mL [*P. aeruginosa*, *S. aureus*, or *C. albicans*] or 5 μg/mL [*A. baumannii*]) and/or caffeine (20 mM [*P. aeruginosa*, *S. aureus*, or *C. albicans*] or 5 mM [*A. baumannii*]) as the inhibitors. All microbial species were cultured aerobically at 37°C in wells of a 96-well microplate (200 μL). The growth of the microbes was monitored at 0 h, 20 h, 45 h, and 70 h [*P. aeruginosa*, *S. aureus*, or *C. albicans*] or at 0 h, 15 h, 25 h, and 40 h [*A. baumannii*] after inoculation by measuring the absorbance of the wells at a wavelength of 590 nm using a MPR-A100 microplate reader (AS ONE Co., Osaka, Japan).

Statistical Analysis All assays were performed as two independent experiments with each consisting of triplicate reactions. Welch's *t* tests were performed to compare independently the data (+$NaClO_2$+caffeine) *versus* data (+$NaClO_2$), *versus* data (+caffeine), or *versus* data (no addition; control). Unless otherwise indicated, values are presented as the mean ± standard deviation.

RESULTS AND DISCUSSION

In the experiments using *P. aeruginosa,* the presence of 100 μg/mL [1.11 mM] $NaClO_2$ considerably suppressed bacterial growth until 20 h after inoculation, but full growth was observed by 45 h after inoculation (Fig. 1A). The addition of 20 mM caffeine alone did not appreciably affect the growth. However, the addition of both 100 μg/mL $NaClO_2$ and 20 mM caffeine halted the growth of *P. aeruginosa.* Ten μL of culture medium containing sodium chlorite and caffeine at 70 h was spread into LBG agar plate, but no colony was recognized.

In the experiments using *A. baumannii*, the presence of 5 μg/mL [55.3 μM] $NaClO_2$ decreased bacterial growth to about one-half that of the untreated control at 15 h after inoculation, but the final yield of bacteria was similar to that of the untreated control at 70 h after inoculation (Fig. 1B). The addition of 5 mM caffeine alone decreased the yield by about one-half that of the untreated control at 70 h. The addition of both 5 μg/mL $NaClO_2$ and 5 mM caffeine completely suppressed the growth of the bacteria. Ten μL of culture medium at 40 h was spread into LBG agar plate, but colony was not observed.

In the experiments using *S. aureus,* the presence of 100 μg/mL $NaClO_2$ appreciably decreased the bacterial growth when compared to that of the untreated control, but the yield had reached almost the same level as that of the untreated control by 70 h after inoculation (Fig. 1C). In contrast, the addition of 20 mM caffeine alone resulted in growth that was similar to or up to 20% higher than that of the untreated control. The addition of both 100 μg/mL $NaClO_2$ and 20 mM caffeine completely inhibited growth. The 70 h-cultured medium (10 μL) containing $NaClO_2$ and caffeine was spread into LBG agar plate, but no colony was grown.

In the experiments using *C. albicans,* the presence of 100 μg/mL $NaClO_2$ had no effect on the growth at 20 h when compared to the untreated control, but the final yield was about 70% to 80% that of the untreated control at 70 h after inoculation (Fig. 1D). The addition of 20 mM caffeine considerably suppressed bacterial growth until 20 h after inoculation, after which recovery of the growth rate was seen, resulting in a yield of about 60% that of the untreated control at 45 h after inoculation. As indicated by the above results, the addition of both 100 μg/mL $NaClO_2$ and 20 mM caffeine resulted in complete growth inhibition. The 70 h-cultured medium (10 μL) in the presence of $NaClO_2$ and caffeine was spread into LBG agar plate, but no colony was indicated.

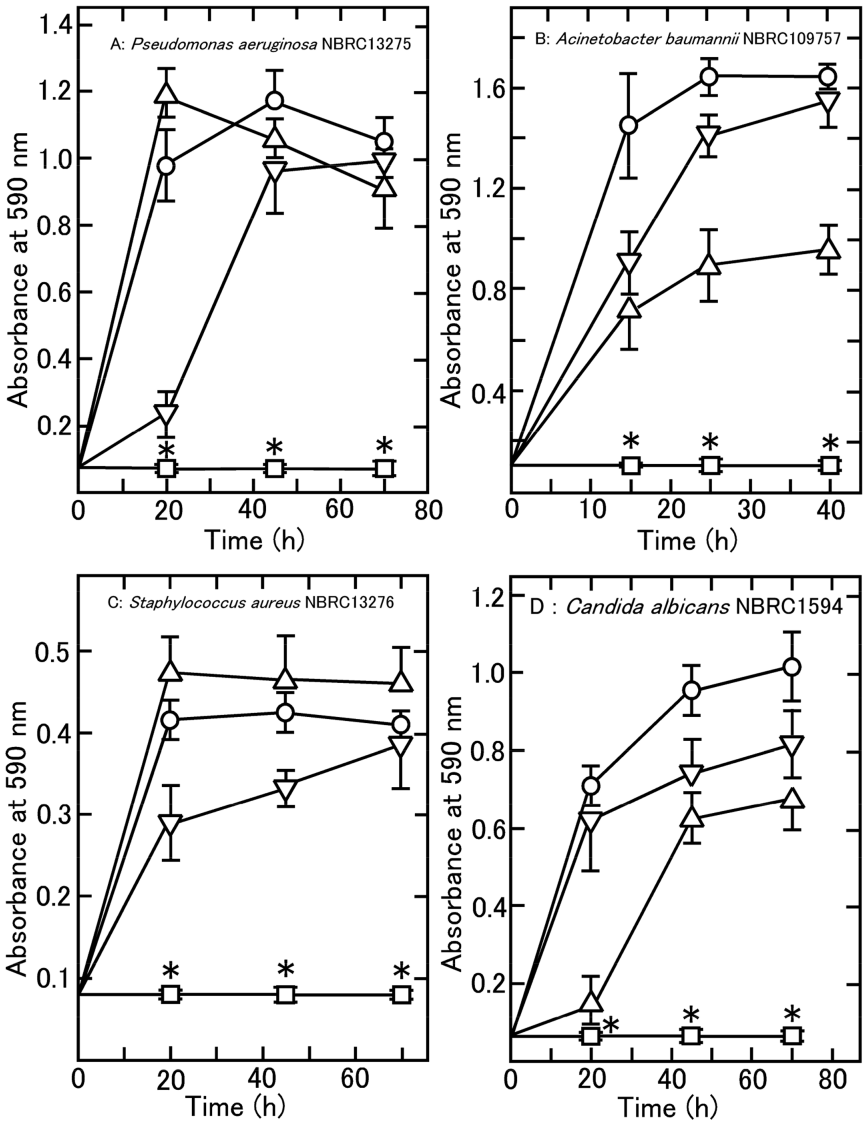

Fig. 1. Inhibitory Effect of the Combination of Sodium Chlorite and Caffeine Against Two Gram-negative Bacteria, a Gram-positive Bacterium, and a Fungus at a Neutral pH.

The four microbial species were cultured aerobically at 37°C in wells of a 96-well microplate (200 µL). The growth of the microbes was monitored by measuring the absorbance of the wells at a wavelength of 590 nm using a microplate reader.

A: *P. aeruginosa* NBRC13275; sodium chlorite--100 µg/mL [1.11 mM]; caffeine--20 mM.

B: *A. baumannii* NBRC109757; sodium chlorite--5 µg/mL [55.3 µM]; caffeine--5 mM.

C: *S. aureus* NBRC13276; sodium chlorite--100 µg/mL; caffeine--20 mM.

D: *C. albicans* NBRC1594; sodium chlorite--100 µg/mL; caffeine--20 mM.

Circles: untreated control; inverted triangles; sodium chlorite; triangles: caffeine; squares: sodium chlorite and caffeine.

Welch's *t* tests were performed to compare independently the data (+$NaClO_2$+caffeine; square) *versus* data (+$NaClO_2$; inverted triangle), *versus* data (+caffeine; triangle), or *versus* data (control; circle), and the data indicated by asterisk at square (+$NaClO_2$+caffeine) were statistically significant differences ($P<0.05$) against the others. Data are shown as the mean ± standard deviation (n = 6).

Although it has been reported that the disinfecting effects of $NaClO_2$ alone are very weak at a neutral pH (pH 7.5), and that caffeine alone also has very slight antimicrobial effects, our results revealed that when used in combination, they had strong antibacterial and antifungal activities against several microbes at a neutral pH (pH 7.5). Additionally, we found that two caffeine analogs, theophylline (1,3-dimethylxanthine) and aminophylline (complex produced from 1 mol of ethylenediamine and 2 mol of theophylline), in combination with $NaClO_2$ exerted a similar inhibitory effect against Gram-negative and Gram-positive bacteria as well as *C. albicans*, indicating a synergistic effect between the two chemicals (data not shown). The mechanism of microbicidal effect of $NaClO_2$ in combination with caffeine is now in progress in our laboratory. A remarkable property of caffeine is its ability to catalyze as a Lewis acid, which is assayed according to the previously published procedure[17] (unpublished observation). We assume that chlorine dioxide would be produced from chlorite ion catalyzed by Lewis acid property of caffeine, and the chlorine dioxide may exhibit a marked antimicrobial activity.

In conclusion, our results indicated that the combination of $NaClO_2$ and caffeine shows potential as a disinfectant.

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Conflicts of Interest Takekatsu Shibata is a director of Acenet Inc., which manufactures the disinfectant MA-T. Another author declares no conflict of interest.

REFERENCES

1) El-Sayed A, Kamel M. Climatic changes and their role in emergence and re-emergence of diseases. *Environ. Sci. Pollut. Res. Int.*, **27**, 22336–22352 (2020).
2) Shibata T, Urakawa R, Sakai T, Konishi K. Bacterial effect of MA-T against *Escherichia coli* in Davis minimal medium in the presence of organic materials, compared to perchlorous acid as a control. *BPB Reports*, **6**, 122–125 (2023).
3) Wei CI, Cook DL, Kirk JR. Use of chlorine compounds in the food industry. *Food Technol.*, **39**, 107–115 (1985).
4) Hinenoya A, Awasthi SP, Yasuda N, Shima A, Morino H, Koizumi T, Fukuda T, Miura T, Shibata T, Yamasaki S. Chlorine dioxide is a better disinfectant than sodium hypochlorite against multi-drug resistant *Staphylococcus aureus, Pseudomonas aeruginosa*, and *Acinetobacter baumannii. Jpn. J. Infect. Dis.*, **68**, 276–279 (2015).
5) Horvath AK, Nagypal I, Peintler G, Epstein IR, Kustin K. Kinetics and mechanism of the decomposition of chlorous acid. *J. Phys. Chem. A*, **107**, 6966–6973 (2003).
6) Horiuchi I, Kawata H, Nagao T, Imaohji H, Murakami K, Kino Y, Yamasaki H, Koyama AH, Fujita Y, Goda H, Kuwahara T. Antimicrobial activity and stability of weakly acidified chlorous acid water. *Biocontrol Sci.*, **20**, 43–51 (2015).
7) Takahashi K, Tanaka R, Fukuzaki S. Kinetics of the inactivation of *Vibrio parahaemolyticus* in weakly acidic sodium chlorite solution. *Biocontrol Sci.*, **22**, 25–30 (2017).
8) Gordon G, Kieffer RG, Rosenblatt DH. The chemistry of chlorine dioxide. In *Progress in Inorganic Chemistry* **15** (Lippard, SJ ed.), pp. 15201-15286. John Wiley & Sons, New York (1972).
9) Ashihara H. Metabolism of alkaloids in coffee plants. *Braz. J. Plant Physiol.*, **18**, 1–8 (2006).
10) Cano-Marquina A, Tarín JJ, Cano A. The impact of coffee on health. *Maturitas*, **75**, 7–21 (2013).
11) Stefanello N, Spanevello RM, Passamonti S, Porciúncula L, Bonan CD, Olabiyi AA, Teixeira da Rocha JB, Assmann CE, Morsch VM, Schetinger MRC. Coffee, caffeine, chlorogenic acid, and the purinergic system. *Food Chem. Technol*, **123**, 298–313 (2019).
12) Raj CVS, Dhala S. Effect of naturally occurring xanthine on bacteria. I. Antimicrobial action and potentiating effect on antibiotic spectra. *Appl. Microbiol.*, **13**, 432–436 (1965).
13) Woziwodzka A, Krychowiak-Maśnicka M, Gołuński G, Łosiewska A, Borowik A, Wyrzykowski D, Piosik J. New life of an old drug: caffeine as a modulator of antibacterial activity of commonly used antibiotics. *Pharmaceuticals (Basel)*, **15**, 872 (2022).
14) Sledz W, Los E, Paczek A, Rischka J, Motyka A, Zoledowska S, Piosik J, Lojkowska E. Antibacterial activity of caffeine against plant pathogenic bacteria. *Acta Biochim. Pol.*, **62**, 605–612 (2015).
15) Hosseinzadech H, Bazzaz BS, Sadati MM. *In vitro* evaluation of methylxanthine and some antibiotics: interaction against *Staphylococcus aureus* and *Pseudomonas aeruginosa. Iran. Biomed. J.*, **10**, 163–167 (2006).
16) Konishi K, Urada M, Adachi I, Tanaka T. Inhibitory effect of sanguiin H-11 on chemotaxis of neutrophil. *Biol. Pharm. Bull.*, **23**, 213–218 (2000).
17) Fukuzumi S, Ohkubo K. Quantitative evaluation of Lewis activity of metal ions derived from the g values of ESR spectra of superoxide: metal ion complexes in relation to the promoting effects in electron transfer reactions. *Chem. Eur. J.,* **6**, 4532-4535 (2000).