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Advanced Study for Defense Medicine

Bioshell Calcium Oxides (BiSCaO) Water used by National Defense Medical College to protect health personnel against COVID-19 infection.

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Summary

1. All health care workers from National Defense Medical College and its hospital who were dispatched to attend to COVID-19 patients on the Diamond Princess cruise ship and at the Osaka Corona Intensive Care Center were well protected from COVID-19 infections with the help of BiSCaO Water.

2. BiSCaO Water is produced from scallop shell and is the first in the world to combine strong sterilization abilities and high levels of safety for humans. BiSCaO is a designated food additive.

3. BiSCaO Water has stronger microbicidal and virucidal activities than ethanol and hypochlorous acid (HOCl).

4. BiSCaO Water can be used by humans for hand sterilization and mouth cleaning without apparent harmful side effects. BiSCaO Water is kind to hands and skin.

5. This article focuses on BiSCaO Water's sterilization ability and safety for infectious cleaning/disinfectant, and to compare with ethanol and HClO on effectiveness and safety to the environment and on humans.

1. Introduction

A division of Biomedical engineering in the Research Institute of National Defense Medical College has been undertaking research and development on a safe and highly versatile protection/ decontamination agents against CBRN (C: hazardous chemicals, B: pathogenic microorganisms,N/R: nuclear/radioactive materials) for more than ten years. The agents researched include metal nanoparticles s uch as silver, copper, zinc oxide, and titanium oxide; weakly acidic hypochlorous acid (HOCI) solution,alcohol, and heated bioshell calcium oxide (BiSCaO®).

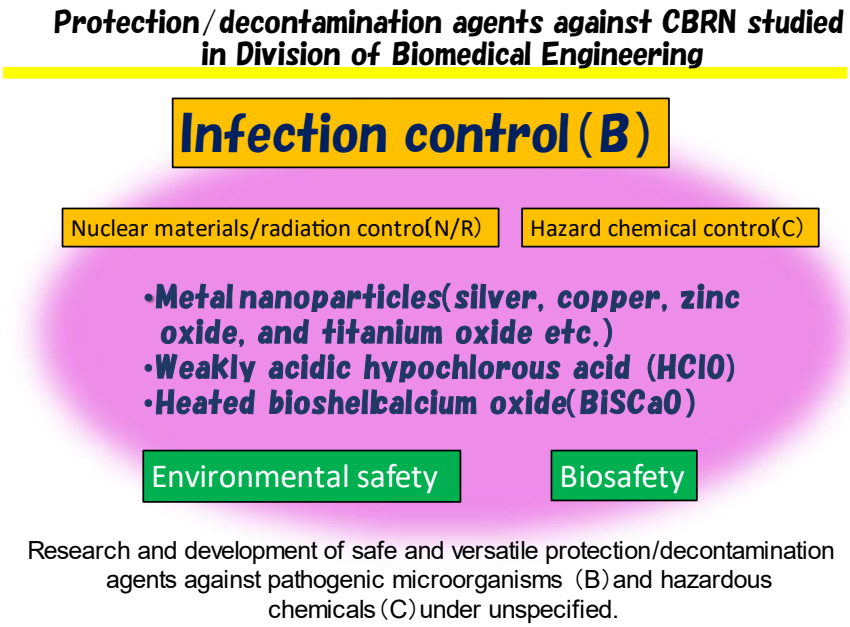

Figure 1. Protection/decontamination agents against CBRN studied in division of Biomedical engineering in Research Institute of National Defense Medical College.

In this current pandemic of coronavirus disease (SARS-CoV-2 which is now called COVID-19), it is evident that some antiseptics/disinfectants, such as ethanol and sodium hypochlorite (NaClO), show significant activity with broad microbicidal and antiviral activities; notably, activity against COVID-19 resulting from disruption of the viral envelope. However, ethanol is flammable and can cause rough hands and rashes. Furthermore, ethanol degrades various chemical fibers, plastic, and non-woven fabrics. Hypochlorous acid (HOCI) solution (pH 6, 200 ppm) is much more superior to sodium hypochlorite (NaClO) solution (pH = 10) in terms of higher microbicidal activity against a broad range of

microorganisms. However, HOCl is less stable to various environmental factors compared to NaClO[1,2]). Notably, the presence of various organic compounds and in-organic ions easily results in rapid deterioration of HOCl. Therefore, low concentration of HOCl (> 50 ppm) under organic matter contaminated environments, high temperature (> 25 ℃), and/or sun shine including UV light rapidly diminishes the microbicidal and antiviral activities of HOCl. Usage of higher concentrations of HOCl (> 100 ppm) are restricted as they cause the generation of hazardous substances including chloramines, trihalomethane, and chlorine (Cl2); and de-colorize and corrodes metal by their oxidizing action. Therefore, disinfectants that can decrease the bacterial and viral bio-burden without harmful side effects and environmental disruption are essential for environmental hygiene and public health.

Calcium oxide (CaO; quicklime) and calcium hydroxide (Ca (OH)2; slaked lime) produced from limestone is readily available and is an important in-organic compound used in various industries, for example, as an absorbent, toxic-waste remediation agent, and an alkalization agent. However, calcined CaO and Ca(OH)2 with fossil fuel contain harmful impurities such as particulate matters, and quicklime has a dangerously high heat of hydration[3,4]) For that reason, direct use to foods and living body is prohibited.

On the other hand, heated scallop or egg shell powder is a readily available source of CaO and Ca(OH)2 which are used as a food additive and environmental hygiene materials. Most scallop shells are considered to be industrial waste, and the shells accumulate on the shores of scallop harvesting districts in Japan, causing serious environmental problems such as offensive odors and soil pollution due to harmful materials such as shellfish poisons and heavy metals leaching from internal organs of discarded scallop shells[5]).

The main component of the most commercially available heated shell powder products as food additives is calcium hydroxide (Ca(OH)2) produced by hydration of CaO, and the effect of Ca(OH2)-based products are lower than other CaO-based products. Bioshell calcium oxide (BiSCaO®) is now commercially available from Plus Lab Co. Ltd., Kanagawa, Japan. Plus Lab performed research and development for BiSCaO® for more than 3 years. According to the product instructions, BiSCaO® contains over 99.6% CaO and 6 μm of average diameter, which is an unprecedented product as cleaning agent /disinfectants[6,7]).

Moreover, BiSCaO Water is a highly concentrated Ca2+ ion solution, is colorless and transparent with pH >12.7. BiSCaO Water is first commercialized for minimizing the spread of infectious diseases following the outbreak of COVID-19 in 2020. This article aimed to introduce BiSCaO ®and BiSCaO Water as a cleaning agent /disinfectant, which we studied and

developed as a subject for advanced study by Defense Medicine in Ministry of Defense during 2017~2019, and to compare it to ethanol and HClO on effectiveness and safety to the environment and the living body.

2. BiSCaO® and BiSCaO Water as useful and safe cleaning agent/disinfectant

２－１. Preparation and characterization of BiSCaO® and BiSCaO Water.

We are studying Biomaterials including suspensions based on BiSCaO® as shown in Figure 2 in Division of Biomedical Engineering of Research Institute in National Defense Medical College. BiSCaO® itself is poorly water-soluble under strongly alkaline conditions. Consequently, BiSCaO® suspensions containing a high concentration of BiSCaO® tend to plug spray nozzles due to precipitation. We previously reported that production and characterization of BiSCaO® dispersion, colloidal dispersion, and BiSCaO Water to prevent the inconvenience[6,7]). BiSCaO Water showed higher deodorization and microbicidal activities than BiSCaO® suspension[6,7]). BiSCaO® is authorized to produce as a food additive for food preservation[8]) and calcium enhancer as well as measures of infectious disease protection and decontamination including COVID-19 by their cleaning/disinfectant and deodorizing properties. Furthermore, we confirmed that BiSCaO® is able to remove coagulation sediment and to decompose and detoxify hazardous chemicals including dioxin such as PCB and water-soluble organophosphorus pesticides.

On the other hand, although in general it is said to be difficult to produce a highly concentrated Ca2+ ion solution under strong alkaline condition, Plus Lab successfully produced a novel BiSCaO Water, prepared by repeatedly (more than 50 times) adding 10 wt% BiSCaO® Powder to chilled clean water and gently decanting the supernatant into a separate container[9,10]). BiSCaO Water is a highly concentrated Ca2+ ion solution, and is colorless and transparent with pH >12.7. BiSCaO Water is commercialized for minimizing the spread of infectious diseases during the COVID-19 outbreak in early 2020. Since then it is widely used by National Defense Medical College and its hospital, elderly care facilities, restaurants, medical institutes, food related companies, and local governments. In fact, a large amount of BiSCaO Water was used for health care workers dispatched from National Defense Medical College and its hospital to the Diamond Princess cruise ship, Osaka Corona Intensive Care Center, and Fever patient center. The result - all dispatched health care

workers were healthy and safe. BiSCaO Water was also used when a corona cluster occurred in a student dormitory of National Defense Medical College. Again, the health workers involved were well protected from being infected with COVID-19 and no side effects were reported to have been suffered. Thus, it has been confirmed that BiSCaO Water is effective and safe.

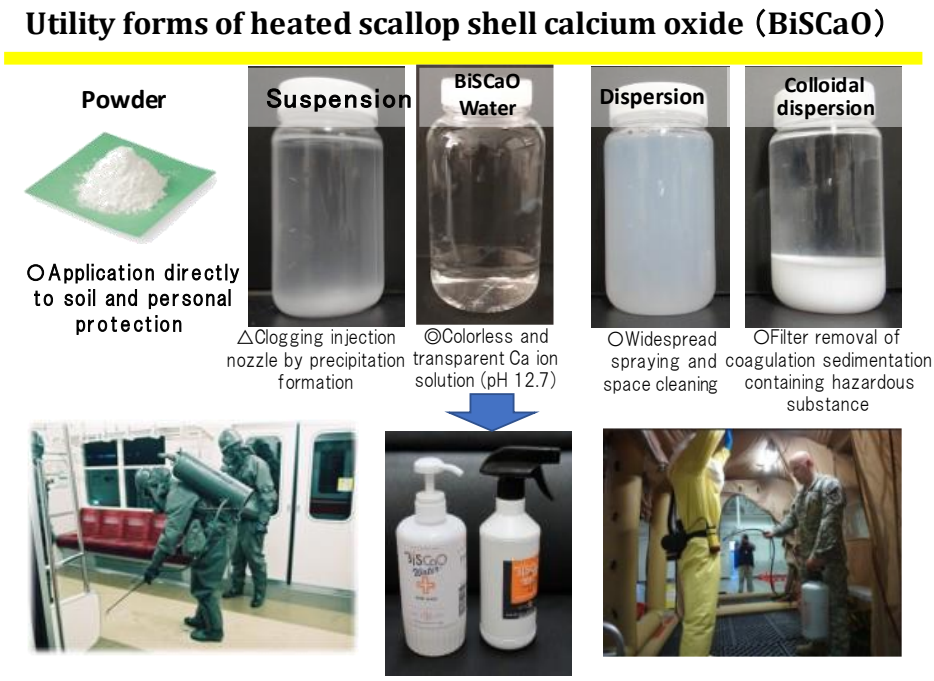

Figure 2. Forms and application of BiSCaO® containing liquids and BiSCaO Water

２－２． Comparison of disinfectant ability between BiSCaO Water, ethanol, and Hypochlorous acid

Disinfection effects of BiSCaO Water[9,10]) against contamination suspensions including 51 ± 14 (× 107) CFU (Colony-Forming Units) /mL of total viable cells (TC) and 45 ± 25 (×10^6) 6 CFU/mL of coliform bacteria (CF) were compared to ethanol and Hypochlorous acid (HClO)[1,2]). Undiluted BiSCaO Water (pH 12.7), ethanol (99.5%), and 200 ppm HClO with commercially available highest concentration (pH 6.2) are defined as 100%, and the mixtures of each disinfectant and contamination suspension with the final concentrations (75, 50, 25, and 12.5%) were tested for microbicidal activities.

The CFU/mL for floating TC and CF with 75 and 50% of BiSCaO Water, ethanol, and HClO were below the detection limit, indicating those three disinfectants have strong microbicidal

activities. The microbicidal activities decreased with lower concentrations of each disinfectant with less than 25% and all the three disinfectants with 6.25% have little microbicidal activities. BiSCaO Water with 25, 12.5, and 6.25% exhibited slightly higher microbicidal activity than ethanol and HClO with no significant difference.

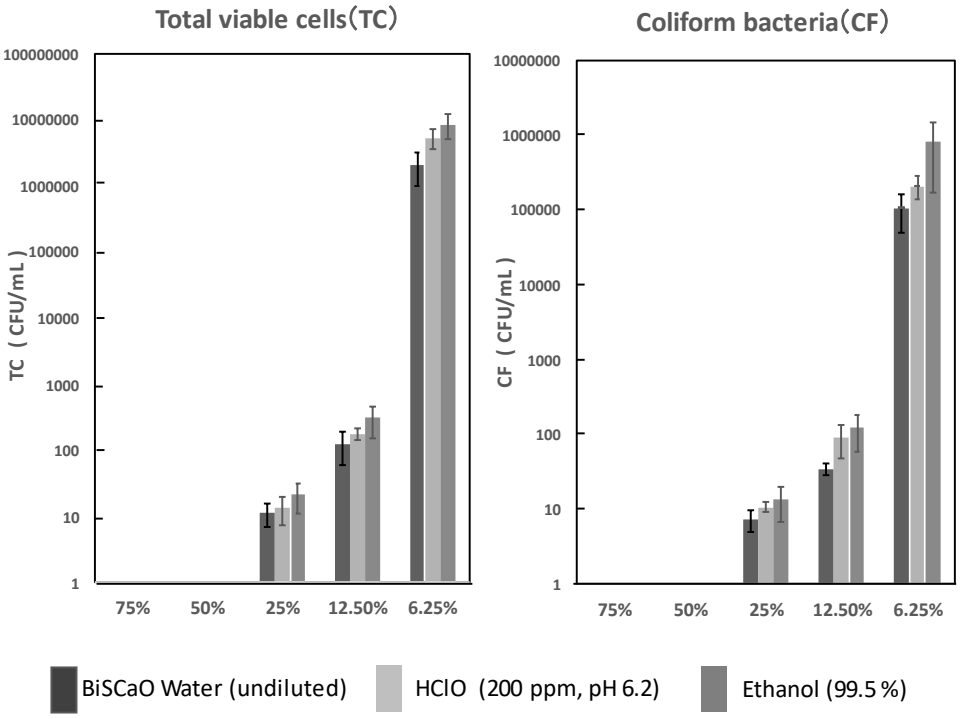

Figure 3. . Bactericidal activity of disinfectants against a contaminated suspension comprising normal bacterial flora. TC (left) and CF (right) released from the contaminated suspension in each sample were measured as CFU/mL. BiSCaO Water, ethanol, and HClO had strong microbicidal activities. Plating and counting were performed as a set of 3 technical replicates (n = 3).

２－３． Comparison of cleaning abilities of BiSCaO Water, ethanol, and HClO to environmental surfaces

Six items - Toilet sandals, flat wood of old wooden box, cowhide sofa, television screen, metal door knob, and acrylic plate for splash prevention (referred to as “environmental surfaces”) were washed with tap water, BiSCaO Water (undiluted), ethanol (75%), and HClO solution (pH 6.2、200 ppm), and then tested with ATP rapid hygiene monitoring (Kikkoman Biochemifa Company, Tokyo) for cleaning effect

and simple wiping. General viable bacteria colony formation test (Nissui Pharmaceutical Co., Ltd.) were performed for cleaning effect and for microbicidal effect, respectively. In this, it is said that optimal concentration of ethanol for disinfection is 70－80%, and commercially available highest concentration of HClO for antiseptic solution is 200 ppm. Therefore, undiluted BiSCaO Water, 75% ethanol, and 200 ppm HClO solution were used in this study.

ATP rapid hygiene monitoring test is to quantify total amount of ATP (adenosine triphasphate), ADP (adenosine diphasphate), and AMP (adenosine monophasphate) as dirt index, based on relative value of chemiluminescence induced with luciferase catalyst (Relative Light Unit or "RLU"). According to the instructions, less than 500 RLU, 501～1000 RLU, and more than 1001 RLU stand for "Clean (Pass)", "Be careful", and "Dirty (Failure)" respectively. As shown in Figure 4, all six items of environmental surfaces before cleaning were Dirty, and all the six items remained as Dirty after cleaning with tap water although RLU values were decreased on television screen, metal door knob, and acrylic plate with tap water. On the other hand, after cleaning television screen, metal door knob, and acrylic plate with BiSCaO Water, ethanol, and HClO - RLU levels decreased to below the detection limit. In other words, they are clean. However, toilet sandals, flat wood of old wooden box, cowhide sofa remained as Dirty (Failure).

From the results of ATP rapid hygiene monitoring, it proven that it is insufficient to wash porous materials such as toilet sandals, flat wood of old wooden box, and cowhide sofa with either BiSCaO Water, ethanol, or HClO. There are no significant differences in RLU values between BiSCaO Water, ethanol, and HClO. Since ATP rapid hygiene monitoring test is to quantify total amount of adenosine phosphate, the data do not reflect effect on removing pathogenic virus and bacteria. Therefore, simple wiping general viable bacteria colony formation test (Nissui Pharmaceutical Co., Ltd.) were performed for microbicidal effect.

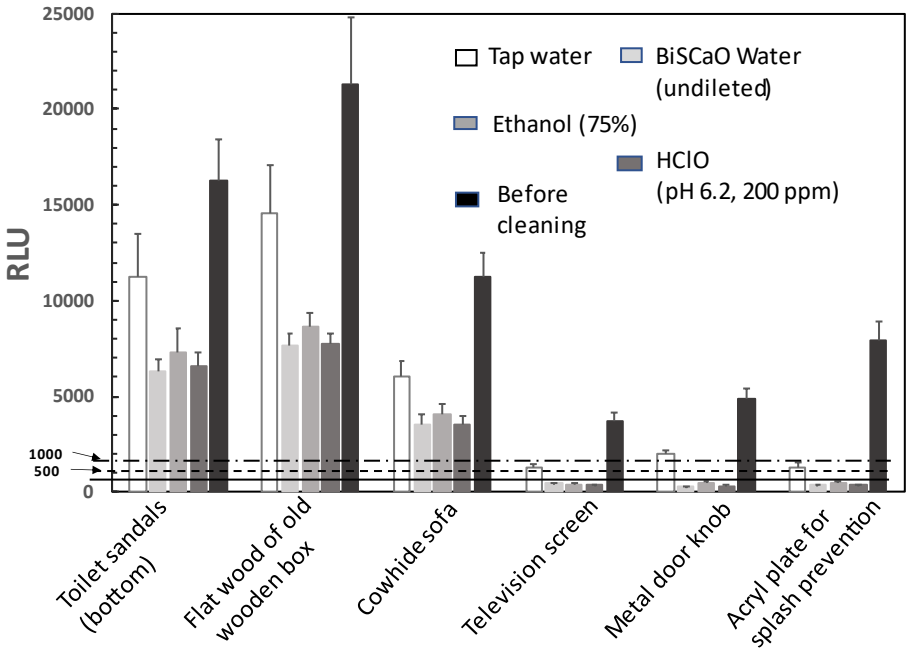

Figure 4. Cleaning effect of BiSCaO Water, ethanol, and HClO by ATP rapid hygiene monitoring test. Each environmental surface after cleaning with either BiSCaO Water, ethanol, or HClO were measured Relative Light Unit (RLU) using ATP rapid hygiene monitoring. The counting was performed as a set of 3 technical replicates (n = 3) and error bars represent means ± S.D.

2－4. Comparison of disinfectant abilities of BiSCaO Water, ethanol, and HClO to environmental surfaces

Simple wiping general viable bacteria colony formation test exhibited that CFU (Colony-Forming Units) /mL of total viable cells (TC) after washing all six environmental surfaces with BiSCaO Water (undiluted), ethanol (75%), and HClO (200 ppm) decreased to less than 100 CFU (Figure 5). Those results suggested that BiSCaO Water, ethanol, and HClO have sufficiently high microbicidal activities in spite of insufficient cleaning ability to porous environmental surfaces by ATP rapid hygiene monitoring. There are no significant differences in RLU values between BiSCaO Water, ethanol, and HClO.

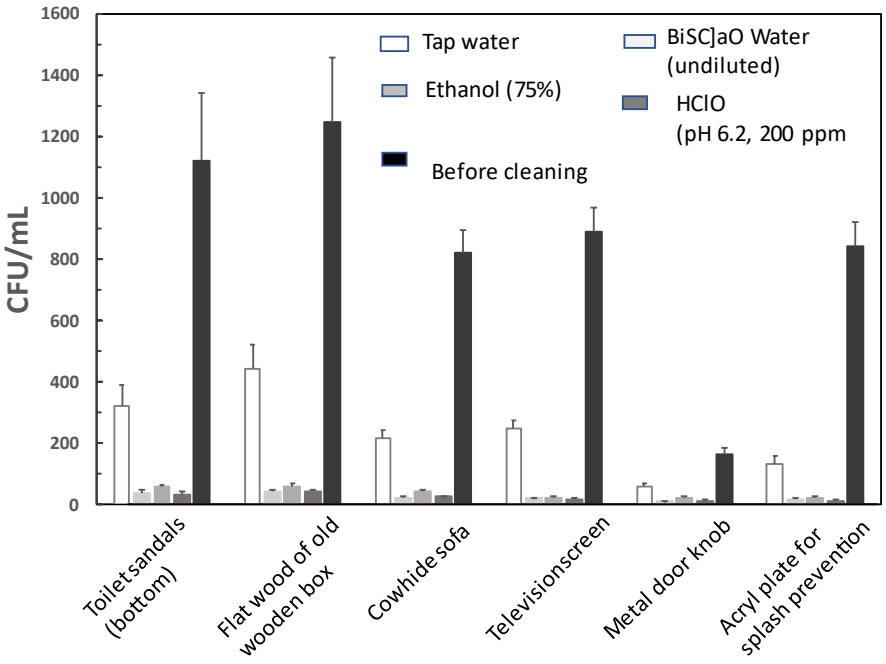

Figure 5. Disinfection effect of BiSCaO Water, ethanol, and HClO by simple wiping general viable bacteria colony formation test. Each environmental surface after cleaning with either BiSCaO Water, ethanol, or HClO were measured CFU (Colony-Forming Units) /mL of total viable cells (TC). The counting was performed as a set of 3 technical replicates (n = 3) and error bars represent means ± S.D.

2－5. Comparison of deterioration in non-woven fabric with BiSCaO Water, ethanol, and HClO[12])

Surgical masks (surgical mask ST; Utsunomiya Seisaku Co. Ltd., Osaka, Japan) and N95 masks (GIKO 1400, NIOSH N95, Fitlife Corp., Tokyo, Japan) were sprayed with BiSCaO Water, ethanol (75%), and hypochlorous acid (200 ppm, pH 6.2) twice per day over 3 days. The Scanning Electron Microscope ('SEM") image of inner sheet in the two BiSCaO Water-treated masks was identical to that of control, which suggests that BiSCaO Water did not cause damage in both masks. In contrast, the inner sheets of both masks were melted by ethanol, and filtration efficiency reduced with ethanol (data not shown). HClO cause minor damages in both masks (Figure 6).

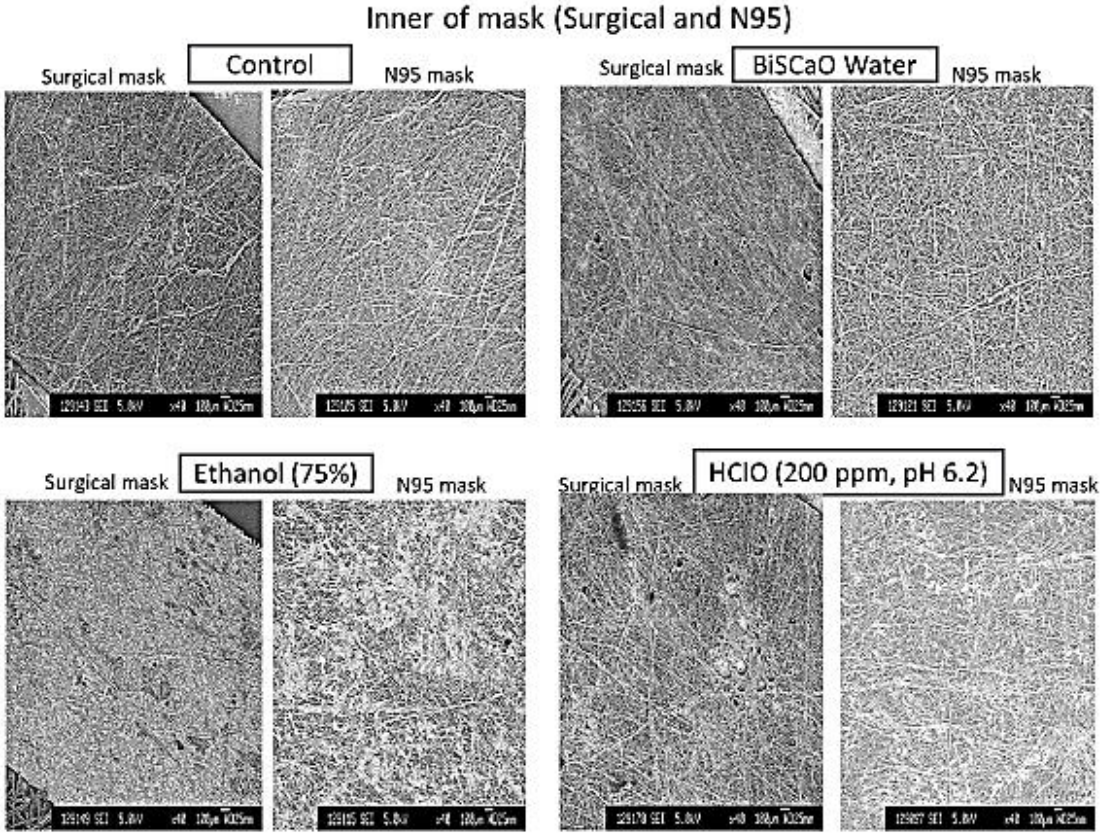

Figure 6. Non-woven fabric deterioration with BiSCaO Water, ethanol, and HClO. The SEM image of inner sheet of surgical and N95 masks, which submerged in enough amount of BiSCaO Water, ethanol, and hypochlorous acid twice per every day for 3 days.

2－6. **Effect of Sterilization of fingers and changes of pH on BiSCaO Water**

Microbicidal effects of BiSCaO Water spray (1.5 mL × 3) to contaminated fingers of three volunteers (a, b, c) were evaluated using Palm Check (Nikken Bio Medical Laboratory Inc. Kyoto) incubated at 37℃ for 24 h (Figure 7 (A)). Although many general viable bacterial colonies were observed before spraying BiSCaO Water, there were no colonies after spraying it. BiSCaO Water was sprayed onto palm, and the pH of wet palm was measured over the course of the next 5 min using a bench pH meter (LAQUA pH meter, F-74, HORIBA, Ltd.) equipped with a Micro Tough electrode (9618S-10D; HORIBA, Ltd.). The pH immediately

decreased after spraying - the pH of the palm fell to 12 after 1 min, and continued falling to weak alkaline and reached to pH 10 after 5 min. Thus, when BiSCaO Water was sprayed onto palm, strong alkaline condition (pH > 12) is maintained for only 1 min. Furthermore, nobody complained of side effects such as rough hands. On the other hand, it has been observed that Sodium Hydroxide (NaOH) solution (pH = 12.7) causes disorders like burns and deteriorates chemical fiber, plastic, and metal such as aluminum and copper. Therefore, NaOH solution may not apply as cleaning/disinfectants.

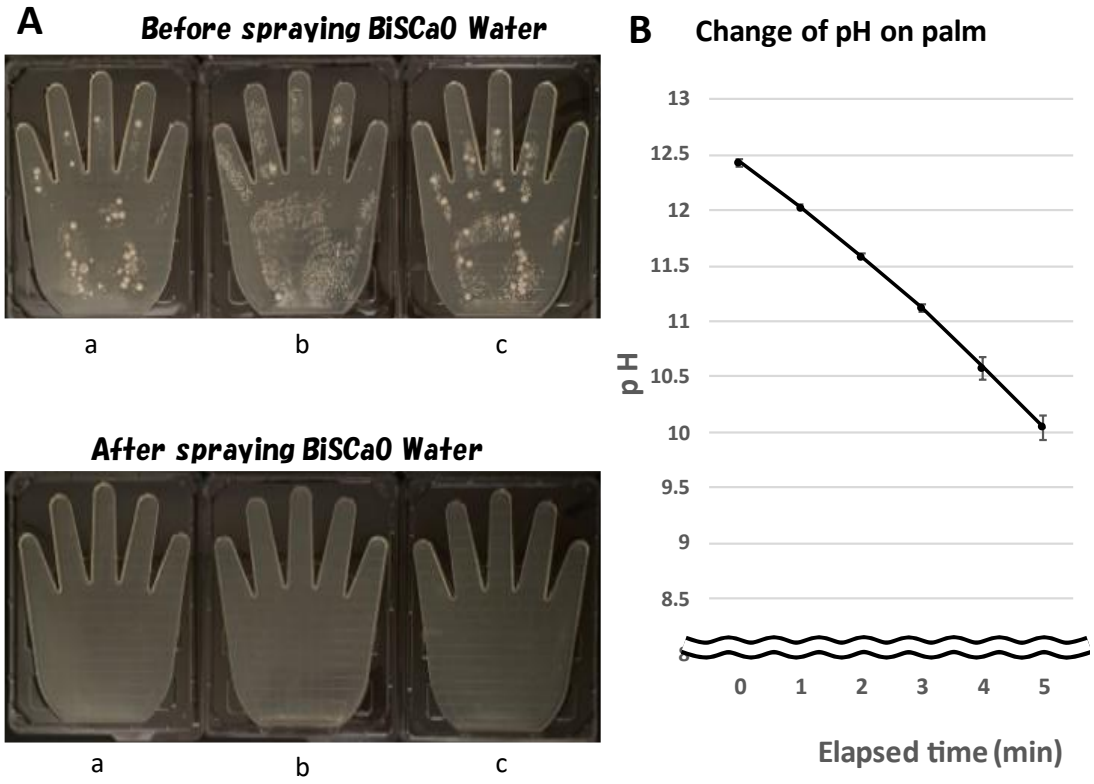

Figure 7. Effect of Sterilization of palms and changes of pH on BiSCaO Water. A: total viable cells before and after spraying BiSCaO Water. B: Changes of pH after spraying BiSCaO Water on palm.

3. Discussion

BiSCaO Water which has strong microbicidal and virucidal activities with strong alkaline (pH 12.7) is produced from scallop shells without alcohol and chlorine-based substances, and is a highly concentrated calcium ion solution. Evaluation by Japan Food Research Laboratory (JFRL) demonstrated that BiSCaO Water by using the fifty-percent tissue culture infectious dose ($TCID_{50}$) method[9] eliminated influenza A (H1N1), an enveloped virus, and Feline calicivirus, a non-enveloped virus within 1 min. Also, by using in vitro assays method, BiSCaO Water eliminated 99% of bacteria such as *Escherichia coli* strains NBRC 3972 and O-157:H7, *Pseudomonas* aeruginosa, Salmonella, and Staphylococcus aureus within 5 mins [9,10] and eliminated 99.9% within 15 mins. Figure 8 summarizes on drug resistances of pathogenic microbes and biological significance of disinfection.

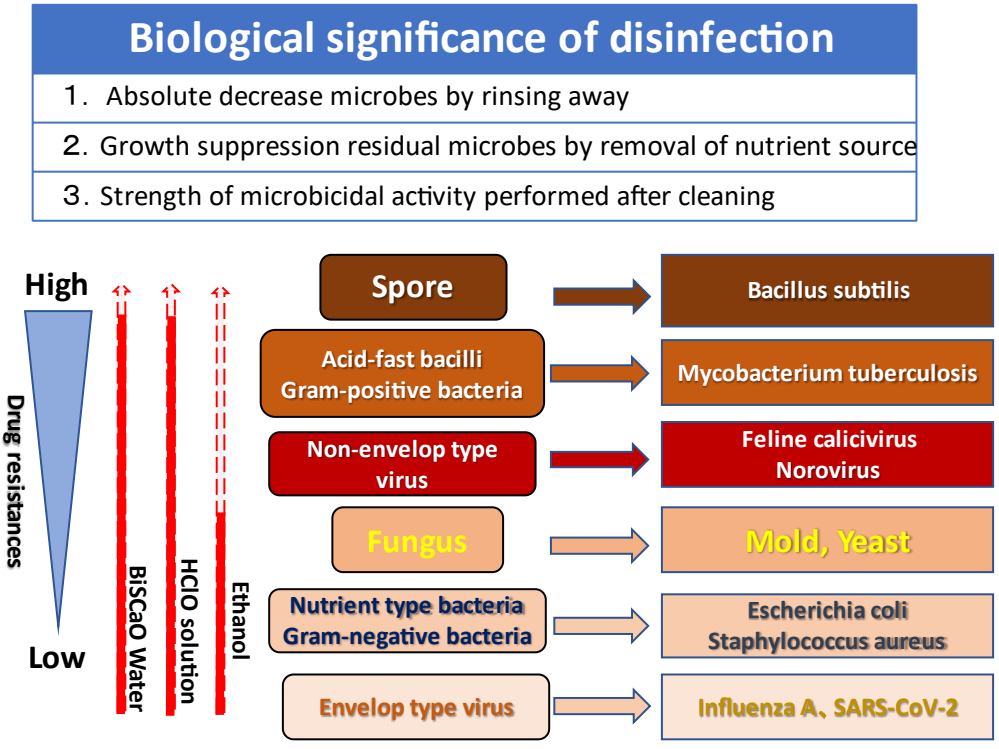

Figure 8. Drug resistances of pathogenic microbes and biological significance of disinfection.

However, since those evaluations were to perform against only solution floating microorganisms, effectiveness of BiSCaO Water against environmental surfaces containing biofilm was not clear. Therefore, we performed study on cleaning and sterilization effects of BiSCaO Water compared/examined to ethanol and HClO. First of all, BiSCaO Water has bactericidal activities identical to ethanol and HClO by the study using contamination suspension (Figure 3). Furthermore, BiSCaO Water exhibited cleaning and sterilization effect against various environmental surfaces identical to ethanol and HClO (Figures 4, and 5). Notably, BiSCaO Water, ethanol, and HClO have sufficiently high microbicidal activities (CFU reduced to tenths of a few) in spite of insufficient cleaning ability (RLU reduced to a fraction) to porous environmental surfaces such as toilet sandals, flat wood of old wooden box, and cowhide sofa by ATP rapid hygiene monitoring. Although there are no significant differences in RLU and CFU values between BiSCaO Water, ethanol, and HClO, BiSCaO Water has slightly higher microbicidal activity than ethanol and HClO (Figures 4, 5).

Some antiseptics/disinfectants, such as ethanol and HClO, show significant activity with broad microbicidal and virucidal activities, notably, activity against COVID-19 resulting in disruption of the viral envelope. However, ethanol has little virucidal activities to non-envelop type virus such as norovirus. On the other hand, chlorine-derived disinfectants are effective to non-envelop type virus, but decolorization and metal corrosive are caused with the effective concentration (> 50 ppm). Furthermore, high concentrations of ethanol and chlorine-derived disinfectant have unique pungent odor. When they directly touch the skin, rough skin and allergies are caused in association with reducing water retention. The direct application of ethanol or HClO to cloth and non-woven masks causes decoloization, melt, and damage. Therefore, disinfectants that can decrease the bacterial and viral bioburden without harmful side effects and environmental disruption are essential for defense and environmental hygiene and public health. Although BiSCaO Water was not directly examined on inactivation of COVID-19, BiSCaO Water can very quickly inactivate both non-enveloped virus such as Feline calicivirus and enveloped virus such as influenza A (H1N1) without harmful side effects and environmental disruption. Since COVID-19 is non-enveloped virus, it can be said that BiSCaO Water is effective to inactivate COVID-19. Furthermore, our study demonstrated BiSCaO Water has identical virucidal activity as cleaning agen/disinfectant for environmental surfaces to ethanol and HClO.

Concerns have been raised on the safety of BiSCaO Water when applied to the living body in view of the strong alkalinity of those reagent. Recent study showed that the high initial pH of BiSCaO Water following application to various environmental surfaces and palm, rapidly decreases to weak alkaline (pH＜10) with the generation of CaCO3 by the interaction between

Ca2+ ions in BiSCaO Water and CO2 in the air[5]. We infer that the ingestion of a small amount of BiSCaO Water would not cause problem if swallowed or inhaled, given that CaCO3 powder will be converted to a safe and soluble compound (calcium bicarbonate (Ca(HCO3)2)) upon further interaction between CaCO3 and CO2.

Moreover, BiSCaO Water has deodorant effect (reduce odor, without masking with fragrance), since it is composed of CaO and Ca ion which are non-volatile[1,2]). Thus, BiSCaO Water is expected to be widely used as an effective and safe decontamination agent for cleaning and sterilizing for various environmental surfaces and finger/palm. In summary, comparisons of BiSCaO Water, ethanol, and HClO on impact to environmental and living body have been described in Table 1 below. Thus, this study on BiSCaO Water suggests a potential application of BiSCaO Water as antiseptic/disinfectant for broad pathological microbes including fungi, bacteria, and viruses, especially for COVID-19 instead of alcohol and chlorine-based disinfectant, and expect to widely apply and use for hygiene activities in Japan Self-Defense Force.

Table 1. Impact of BiSCaO Water on the environment and living organisms

	BiSCaO Water (Undiluted)	Ethanol (75%)	HClO (pH 6.2, 200 ppm)
Impact on environment	+ Little odorless, non-irritating, metal corrosive, anddecolorization. + Generating no hazardous substances and easy wastewater treatment. + Easy thermal control inhydration. + Rapidly decreases to weak alkaline-line (pH＜10) with the generation of $CaCO_3$ by the interaction bet-ween Ca^{2+} ions and CO_2 in the air.	+ Significant alcohol pungent odor and less metal corrosive + Generating little hazardous substances and easy waste water treatment. + No fires for flammability. + The direct application of ethanol or HClO to cloth and nonwoven masks causes melt,decoloization, and damage. + Limitation of use by halal.	+ Irritation, metal corrosive, decolorization for strong oxidizing action, and chlorine odor generated by interaction with organic substances. + Hazardous substances such as trihalomethane and chloramine are generated and difficult wastewater treatment. + High environmental load.
Impact on living organisms	+ A potential application of BiSCaO Water as antiseptic / disinfectant for broad , pathological microbes including fungi, bacteria, and viruses, especially for COVID-19. + Directly touch the skin never causes rough skin and allergies in association without reducing skin-water retention. + No hazardous substance.	+ Inactive to non-envelope type virus such as norovirus. + Directly touch the skin never causes rough skin and allergies in association with reducing skin water retention. + No hazardous substance. +Bleaching/disassembling chemical fiber, non-woven mask, and oil-based ink etc. + Limitation of use by halal.	+ a potential application of BiSCaO Water as antiseptic/disinfectant for broad pathological microbes including fungi, bacteria, and viruses, especially for COVID-19. + Directly touch the skin never causes rough skin and allergies. + Generation of hazardous substances such as trihalomethane and chloramine are generated and generation of chlorine gas.

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