International Journal of Nanomedicine

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ORIGINAL RESEARCH

Bactericidal activities of woven cotton and nonwoven polypropylene fabrics coated with hydroxyapatite-binding silver/titanium dioxide ceramic nanocomposite "Earth-plus"

This article was published in the following Dove Press journal:
International Journal of Nanomedicine
8 September 2011
Number of times this article has been viewed

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Background: Bacteria from the hospital environment, including linens and curtains, are often responsible for hospital-associated infections. The aim of the present study was to evaluate the bactericidal effects of fabrics coated with the hydroxyapatite-binding silver/titanium dioxide ceramic nanocomposite "Earth-plus".

Methods: Bactericidal activities of woven and nonwoven fabrics coated with Earth-plus were investigated by the time-kill curve method using nine bacterial strains, including three *Staphylococcus aureus*, three *Escherichia coli*, and three *Pseudomonas aeruginosa* strains.

Results: The numbers of viable *S. aureus* and *E. coli* cells on both fabrics coated with Earth-plus decreased to below 2 $\log_{10}$ colony-forming units/mL in six hours and reached the detection limit in 18 hours. Viable cell counts of *P. aeruginosa* on both fabrics coated with Earth-plus could not be detected after 3–6 hours. Viable cells on woven fabrics showed a more rapid decline than those on nonwoven fabrics. Bacterial cell counts of the nine strains on fabrics without Earth-plus failed to decrease even after 18 hours.

Conclusion: Woven cotton and nonwoven polypropylene fabrics were shown to have excellent antibacterial potential. The woven fabric was more bactericidal than the nonwoven fabric.

Keywords: hydroxyapatite, silver, TiO_2, Earth-plus, ceramic, bactericidal, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*

Introduction

Prevention of infections associated with health care is extremely important, not only for the safety of health care institutions but also for financial reasons. There are many immunocompromised patients in hospitals and adequate infection control measures are necessary. Many pathogenic microorganisms including bacteria, fungi, and viruses, cause hospital-related infections. Among them, methicillin-resistant *Staphylococcus aureus*, multidrug resistant *Pseudomonas aeruginosa*, and bacteria with extended-spectrum β-lactamase are significant problems.[1–5]

The prevention of cross-infection is a serious issue in medical care and welfare institutions. Although the standard precautions and maximal barrier precautions are very important and performed to diminish cross-infections, these procedures are not sufficient to completely prevent the incidence of infection. Reduction of pathogenic bacteria in the patient environment, which may exist on door knobs, handrails, faucets, tables, beds, linen, clothes, curtains, and the like, is an important medical practice to prevent hospital-related infections and health care-associated infections.[6–13]

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http://dx.doi.org/10.2147/IJN.S24280

Medical devices pose a great risk of cross-infection because bacteria easily attach to these devices and can therefore be transmitted to the patients.[14–19] Metals such as silver (Ag), copper, and titanium dioxide (TiO_2) are known to have an antimicrobial effect, and Ag is commonly used to prevent bacterial contamination in urinary tract catheters and endotracheal tubes.[7,8,15,20] However, the antimicrobial effect of Ag is poor in an environment devoid of water. TiO_2, on the other hand, is a photocatalyst in the oxidation of harmful substances such as microbes, molds, odors, and soils, and TiO_2 is effective in both wet and dry environments.[9,21,22]

The new hydroxyapatite-binding Ag/TiO_2 ceramic nanocomposite, "Earth-plus", was developed by the Shinshu Ceramics Co Ltd, Nagano, Japan. Earth-plus can be coated on various materials, including fabrics (curtains, linen, clothes, and socks), metals, and plastics commonly found in the health care environment and in welfare institutions. Several products using Earth-plus are available on the market, ie, an air purifier with Earth-plus-coated filters,[23] a water-purifier tank with Earth-plus-coated plates, and a mask coated with Earth-plus.[24] Earth-plus obtained a Japanese patent in 1998.[25]

The objective of the present study was to evaluate the bactericidal properties of fabrics coated with Earth-plus against *S. aureus*, *Escherichia coli*, and *P. aeruginosa* strains, which are commonly encountered as causative pathogens of hospital-related infections.

Materials and methods

Woven cotton fabric and nonwoven polypropylene fabric were used in the study. The woven and nonwoven fabrics were coated with Earth-plus (4.5 g/m^2 and 6.0 g/m^2, respectively) using the dipping method developed by Shinshu Ceramics (Nagano, Japan). In summary, fabrics were soaked in a processing liquid containing Earth-plus. Although the concentrations of Earth-plus in the liquid are determined by the processed fabrics and the planned adhesion capacity, the final amounts used were 7 wt% of Earth-plus for woven fabrics and 10 wt% of Earth-plus for nonwoven fabrics. After a specific period of immersion in the liquid, the extra liquid was squeezed out by pressing the fabrics with a roller, and the fabrics were then dried at 160°C for three minutes for woven fabrics and 130°C for one minute for nonwoven fabrics. Woven and nonwoven fabrics without Earth-plus coating and fabrics coated only with hydroxyapatite were also examined as controls. The fabrics were cut into 3 cm squares and then sterilized by autoclaving at 121°C for 20 minutes.

A total of nine strains, which consisted of three strains of *S. aureus*, three of *E. coli*, and three of *P. aeruginosa,* were used for evaluating the bactericidal activities of the woven and nonwoven fabrics coated with Earth-plus. The bacteria used were three standard strains of *S. aureus* ATCC29213, *E. coli* ATCC25922, and *P. aeruginosa* ATCC9027 and six clinical strains, including methicillin-susceptible *S. aureus*, methicillin-resistant *S. aureus*, extended-spectrum β-lactamase-producing and -nonproducing *E. coli*, and metallo-β-lactamase producing and nonproducing *P. aeruginosa* isolates. The latter six strains were clinically isolated from patients at Shinshu University Hospital. They were stored in Micro-Bank vials (Pro-Lab Diagnostics, Ontario, Canada) at −83°C in a deep freezer after identification by the MicroScan WalkAway (Dade Behring Inc, Deerfield, IL) system.

The preparation of bacterial suspensions was performed following the procedures recommended by the Japanese Industrial Standards L1902.[26,27] Prior to the examinations, the nine strains were grown on heart infusion agar (Eiken Chemical Co, Osaka, Japan) plates at 35°C for 24 hours. The colonies on the plates were then inoculated into heart infusion broth (Eiken Chemical Co) media and incubated with gentle agitation at 35°C for 18 hours. The bacterial suspensions were diluted with sterilized physiological saline solution to adjust to a final density of approximately 1×10^5 colony-forming units (CFU)/mL.

The time-kill curve procedure recommended by the Japanese Industrial Standards L1902[26,27] was used in this study. Each fabric was aseptically placed into sterilized glass containers with 50 mL capacity. A bacterial suspension of nine isolates (100 μL, approximately 10^4 CFU/mL) was dropped onto each fabric and incubated at 35°C under ambient air. Bacterial solutions were extracted from each fabric by heavy shaking 30 times and used for viability measurements at various time points, namely, hours 0, 1, 3, 6, and 18. Viability was determined by the plate-colony count method. After 10-fold serial dilutions with sterilized physiological saline solution, 100 μL of each sample was plated onto heart infusion agar plates. Colonies were counted after incubation for 24 hours at 35°C. Measurements were performed in triplicate.

Morphology of bacterial cells on the fabrics

After 18 hours of incubation, the fabrics were cut into small pieces and placed in 1% glutaraldehyde solution for scanning electron microscopy. The specimens were stored in 1% cacodylate buffer containing 1% glutaraldehyde and 4% formaldehyde at 4°C. The fixation solutions were rinsed out with cacodylate buffer and the samples were immersed in 1% osmium tetroxide in cacodylate buffer for two hours. After rinsing, the

specimens were dehydrated by increasing concentrations of acetone. After placing the specimens on electron microscopy plates, they were coated with 200 Å gold (Bio-Rad, SEM coating system Hercules, CA), and electron microscopy was performed using a JSM-6360 LV scanning electron microscope (JEOL, Tokyo, Japan) at 15 kV of accelerating voltage.

Silver ion concentration in the suspensions

The Earth-plus-coated woven and nonwoven fabrics were cut into 5 cm squares, and then ten pieces of each fabric were immersed in 350 mL of ultrapure water at room temperature. After a standing time of 120 hours, the incubation water was centrifuged (10,000 rpm for 60 minutes), and the supernatant was analyzed to determine the amounts of Ag derived from the fabrics using inductively coupled plasma mass spectrometry with the SPQ9200 analyzer (Seiko Instruments Inc, Chiba, Japan).[28,29]

Statistical analysis

Data were plotted as the mean ± standard error of mean. Viable bacterial cell counts were analyzed by the Mann–Whitney *U* test. A *P* value of <0.05 was considered statistically significant.

Results

Evaluation of bactericidal activities

Time-kill curves of the average counts of three *S. aureus* strains on the woven and nonwoven fabrics are shown in Figure 1; the three strains showed similar curves. Live bacterial cell counts on both the woven and nonwoven fabrics coated with Earth-plus gradually decreased to below 2-$\log_{10}$ CFU/mL in six hours and reached the detectable limits of the method in 18 hours. The counts on the woven fabric coated with Earth-plus decreased more rapidly than those on the nonwoven fabric without Earth-plus ($P = 0.036$ in one hour and $P = 0.0140$ in three hours). The counts on the woven and nonwoven fabrics without Earth-plus did not decrease during the 18 hours of incubation.

Time-kill curves of the average counts of three *E. coli* strains on the woven and nonwoven fabrics are shown in Figure 2; the three strains showed similar curves. Live bacterial cell counts on both woven and nonwoven fabrics coated with Earth-plus gradually decreased to below 2-$\log_{10}$ CFU/mL six hours after inoculation and reached detectable limits in 18 hours. The counts on the woven fabric with Earth-plus showed a more rapid decrease than those on the nonwoven fabric, but this difference did not reach statistical significance. The counts on the woven and nonwoven fabrics without Earth-plus did not decrease during 18 hours of incubation.

Time-kill curves of three strains of *P. aeruginosa* on the woven and nonwoven fabrics are shown in Figure 3. Live bacterial cell counts on both woven and nonwoven fabrics coated with Earth-plus gradually decreased and reached detectable limits six hours after the inoculation. However, the time-kill curves showed an upward slope at 6–18 hours due to the increase in cell counts of two of the three strains, namely, the ATCC strain and the strain without metallo-β-lactamase. The counts on the woven fabric with Earth-plus decreased more rapidly than those on the nonwoven without Earth-plus ($P = 0.0011$ in one hour). The counts on the woven and nonwoven fabrics without Earth-plus showed a gradual increase during the incubation

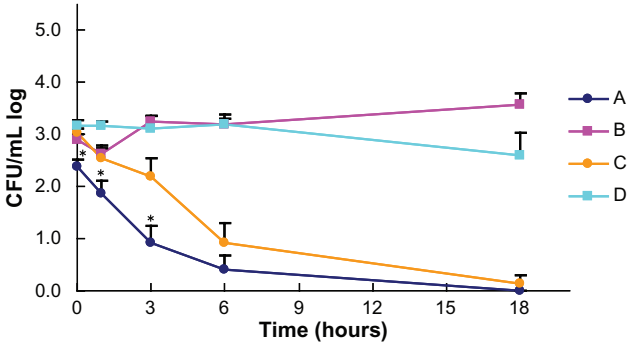

Figure 1 Bactericidal activity against *Staphylococcus aureus* of woven and nonwoven fabrics with and without Earth-plus. Three *S. aureus* strains were incubated with the woven cotton fabric coated with Earth-plus (**A**), woven cotton fabric without Earth-plus (**B**), nonwoven polypropylene fabric coated with Earth-plus (**C**), and nonwoven polypropylene fabric without Earth-plus (**D**). Samples were taken at the indicated times, and viability was determined by colony counting. The bactericidal activities of the woven fabrics with Earth-plus were significantly more rapid than those of the nonwoven fabrics.
Note: *$P < 0.05$; Mann–Whitney *U* test.

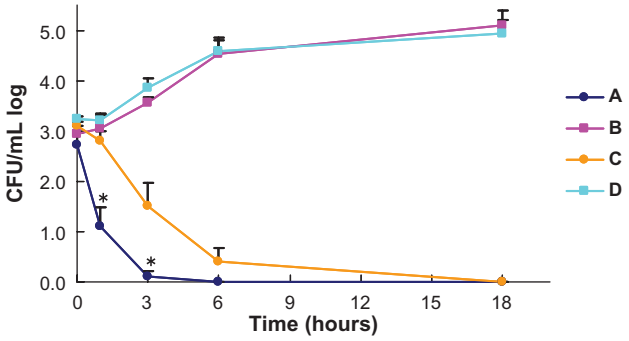

Figure 2 Bactericidal activity against *Escherichia coli* of woven and nonwoven fabrics with and without Earth-plus. Three *E. coli* strains were incubated with woven cotton fabric coated with Earth-plus (**A**), woven cotton fabric without Earth-plus (**B**), nonwoven polypropylene fabric coated with Earth-plus (**C**), and nonwoven polypropylene fabric without Earth-plus (**D**). Samples were taken at the indicated times, and viability was determined by colony counting. The bactericidal activities of the woven fabrics with Earth-plus were significantly more rapid than those of the nonwoven fabrics.
Note: *$P < 0.05$; Mann–Whitney *U* test.

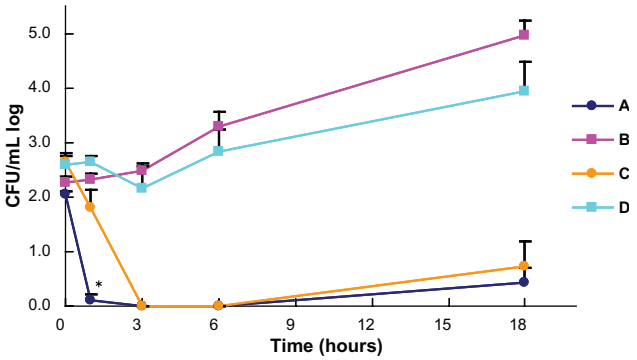

Figure 3 Bactericidal activity against *Pseudomonas aeruginosa* of woven and nonwoven fabrics with and without Earth-plus. Three *P. aeruginosa* strains were incubated with woven cotton fabric coated with Earth-plus (**A**), woven cotton fabric without Earth-plus (**B**), nonwoven polypropylene fabric coated with Earth-plus (**C**), and nonwoven polypropylene fabric without Earth-plus (**D**). Samples were taken at the indicated times, and viability was determined by colony counting. The bactericidal activities of the woven fabrics with Earth-plus were significantly more rapid than those of the nonwoven fabrics.
Note: *$P < 0.05$; Mann–Whitney *U* test.

period. *E. coli* and *P. aeruginosa* were killed more rapidly than *S. aureus* on both fabrics coated with Earth-plus. Preliminary examination of bactericidal activities of fabrics coated only with hydroxyapatite showed no changes in the bacterial counts of *S. aureus* ATCC29213, *E. coli* ATCC25922, and *P. aeruginosa* ATCC9027 after six and 18 hours of incubation compared with the fabrics without Earth-plus.

Concentration of silver ions

Silver ions derived from both Earth-plus-coated fabrics were eluted at a concentration of around 0.2 parts per billion.

Scanning electron microscopy analysis

Morphological observations of the three species after six hours of exposure to the woven fabrics coated with Earth-plus are shown in Figures 4–6. *S. aureus* ATCC29213 cells attached to the Earth-plus granules (Figure 4) mostly showed a normal round shape. However, some cells showed alterations in shape and an irregular surface.

E. coli ATCC25922 cells also attached to the Earth-plus granules and exhibited alterations in shape, with a partially depressed surface (Figure 5). The cells of *P. aeruginosa* ATCC25922 (Figure 6) showed similar alterations in shape as *E. coli.* Cell debris or crushed cells were not observed.

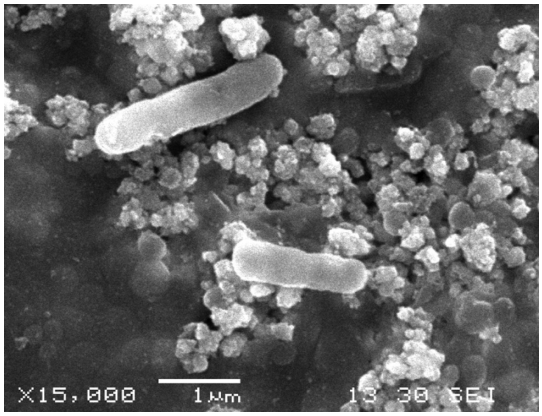

Figure 5 *Escherichia coli,* scanning electron microscope. *E. coli* cells attached to the Earth-plus granules showed a rod shape with a partially depressed surface.

Discussion

Woven cotton and nonwoven polypropylene fabrics coated with the hydroxyapatite-binding Ag/TiO_2 ceramic nanocomposite, Earth-plus, showed bactericidal effects against *S. aureus*, *E. coli*, and *P. aeruginosa*, which are frequent causative pathogens in hospital-related and health care-associated infections. Earth-plus consists of fine granules that can be used to coat many materials, including fabrics (curtains, linen, clothes, and socks), metals (door knobs, faucets, and

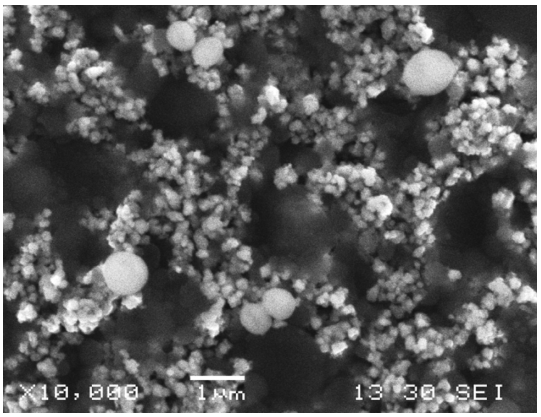

Figure 4 *Staphylococcus aureus*, scanning electron microscope. *S. aureus* cells attached to the Earth-plus granules showed a round shape with a partially depressed surface.

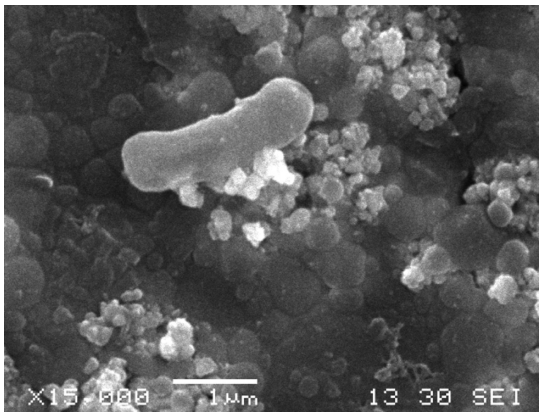

Figure 6 *Pseudomonas aeruginosa,* scanning electron microscope. *P. aeruginosa* cells attached to the Earth-plus granules showed a rod shape with a partially depressed surface.

bed rails), and plastics (keyboards, television controllers, and switches). The use of Earth-plus in hospital rooms and wards can potentially reduce the number of bacterial pathogens in the patient environment, thus preventing cross-infection among patients and health care workers.

Several composite materials consisting of hydroxyapatite-binding Ag and TiO_2 have been developed and evaluated for their antimicrobial activities.[22,30–32] However, these materials have been difficult to convert into a commercially available product. We developed Earth-plus as a hydroxyapatite-binding Ag and TiO_2 composite and showed that it can be used for coating different materials such as woven and non-woven fabrics by using a dipping method. Several products with the Earth-plus technology are currently available on the market, including an air purifier, a water-purifier tank, and a mask.[23,24] Earth-plus obtained a Japanese patent in 1998.[25] Curtains coated with Earth-plus have been used in Nagano Kiso Hospital in Japan for three years, and the properties of Earth-plus on these curtains have not shown changes after washing (data not shown). The cost of curtains coated with Earth-plus was estimated to be 10%–50% higher than that of uncoated curtains.

Slight to moderate morphological changes in the bacterial cells were observed on the Earth-plus-coated fabrics using scanning electron microscopy, but severely damaged cells and cell debris could not be found. Although we initially assumed that bacterial cells in various stages of cell death would be detected on the fabric, only cells showing early stages of damage were imaged. This could be due to the detachment of cells from the Earth-plus during the early stages of cell damage.[33,34]

Among the three species investigated, the Gram-negative pathogens *E. coli* and *P. aeruginosa* were killed more rapidly than the Gram-positive *S. aureus* pathogens, which could be due to differences in cell structure between Gram-negative and Gram-positive bacteria,[35,36] namely, the increased thickness of the cell wall of Gram-positive bacteria compared with that of Gram-negative bacteria. This could result in a longer period of time required for the Earth-plus to kill the Gram-positive bacteria. Moreover, the negative charge on the surface of Gram-negative bacterial cells could have resulted in a stronger attraction to the Earth-plus material.

Interestingly, the time-kill curves corresponding to the *P. aeruginosa* strains showed an upward slope during 18 hours of incubation. In particular, *P. aeruginosa* ATCC9027 and a clinical metallo-β-lactamase-nonproducing strain slightly and gradually proliferated in 18 hours, while the metallo-β-lactamase-producing clinical isolate failed to regrow. This is likely due to the fact that *P. aeruginosa* ATCC9027 and the clinical strain are capable of forming biofilms, which may have been protected from the effect of Earth-plus. Drug-resistant metallo-β-lactamase-producing clinical *P. aeruginosa* isolates with plasmids show a reduced ability to form biofilms. Resistant bacteria that have acquired extracellular drug-resistance genes generally show weaker viability than sensitive strains.[37–39] The ability of the *P. aeruginosa* ATCC9027strain to form biofilms is therefore thought to be one of the reasons for the regrowth. These data have implications for the control of infection in hospitals, where drug-resistant bacteria are commonly found.

The Ag ion concentration measured after washing fabrics coated with Earth-plus was approximately 0.2 ppb, which is below the antimicrobial concentration of 50 ppb.[29] The bactericidal effect of Earth-plus itself was therefore not affected by Ag ions eluted from the material. The antimicrobial activity of Ag is dependent on Ag cations that can be eluted in water and strongly bind to electron donor groups in biological molecules containing sulfur, oxygen, or nitrogen atoms.[29,40–42] Although the antimicrobial activity of Ag has been utilized extensively in medicine, one major problem is its poor performance in environments devoid of water, in addition to the gradual decrease in the activity of Ag over time.

TiO_2, on the other hand, generates strongly oxidizing substances against microbes, moulds, or odors under visible-light irradiation, and it degrades or decomposes them into carbon dioxide, water, and/or other smaller molecules.[9,21,22] The energy from photons generates an electron-hole pair on the TiO_2 surface, which reacts with hydroxide ions (OH^-) in water to yield a hydroxyl radical (OH^+). In addition, the electron in the conduction band can reduce O_2 to produce superoxide anions (O_2^-). Both the OH^+ and the O_2^- are extremely reactive against organic compounds and promptly transform them into nontoxic materials. TiO_2 is one of the most promising photocatalysts, not only in water environments but also under dry conditions.[43–45] The TiO_2 materials only behave as an accelerator without altering their structure, and theoretically, their catalytic activities should last indefinitely.

TiO_2 requires ultraviolet light at wavelengths of less than 385 nm to acquire a strong antimicrobial effect, and it therefore has no activity in the human body in the absence of visible-light illumination,[7,46,47] which hinders its practical application. TiO_2 can photochemically decompose not only microorganisms but also organic materials such as organic paint, textiles, and paper.[22] The antimicrobial activity of TiO_2 requires specific conditions in the environment.

Apatite has been used in chromatographic columns to selectively adsorb protein and in respirators for selective adsorption of pollen.[22,48–50] Hydroxyapatite is responsible for the chemical reaction by which microorganisms are adsorbed and trapped.[25,37,38,40] The adsorption and immobilization of viruses or bacteria on hydroxyapatite facilitates their decomposition through a photocatalytic process mediated by TiO_2. Microorganisms adsorbed by hydroxyapatite are not released except through strong shaking or hard vibration. We confirmed that heavy shaking with an excess of 30 repetitions was necessary to release the bacterial cells trapped on the hydroxyapatite (data not shown). Decomposition through the TiO_2 photocatalytic process is only possible with the excitation of the Ag ion, which enhances the electron-hole separation and interfacial charge transfer.[32,35,41–43]

The photocatalytic activity of TiO_2 becomes weak when it coexists with hydroxyapatite.[31] Therefore, the activity of TiO_2 can be adjusted by modifying the amount of hydroxyapatite. The bactericidal activity of Earth-plus can therefore be explained as follows. Once microorganisms are adsorbed by hydroxyapatite, they cannot be released and are destined to be killed by decomposition through the TiO_2 photocatalytic and Ag bactericidal processes.

Earth-plus can be used to coat most materials found in hospital rooms and wards, and various species of bacteria are killed without antibiotics and disinfectants simply by coming into contact with Earth-plus on doorknobs, tables, walls, and linen, which makes it an effective agent for the control of bacterial pathogens found in dry environments. The effect of Earth-plus was found to have a time lag of several hours, which indicated a weak effect. However, the attenuation of its effect by adjustment of the proportion of hydroxyapatite is necessary to make Earth-plus harmless to humans.

The effective control of causative bacteria in the proximity of patients will reduce cross-infections in hospitals and health care institutions. Causative bacteria are directly transmitted between patients and by medical workers through hands and/or medical instruments, and they are also spread to patients and medical workers via the patient environment. Earth-plus can decrease the latter course of bacterial transmission. A hospital room with Earth-plus-coated equipment, including curtains, linen, and wallpaper, and air cleaners with Earth-plus-coated filters will be built in Japan to test the antimicrobial effects of this material.

Disclosure

The authors have no conflicts of interest to disclose in relation to this work.

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