Antimicrobial Effect of an Ultrasonic Levitation Washer Disinfector with Silver Electrolysis and Ozone Oxidation on Methicillin-Resistant *Staphylococcus aureus* (MRSA)

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Running head: Effect of Levitation with Ag^{+} and O_3 on MRSA

Abstract

Background: Methicillin-resistant *Staphylococcus aureus* (MRSA) has rapidly emerged as a cause of severe and intractable skin infection. At present there are no effective topical treatments and infection or colonization by MRSA of the skin raises serious medical problems. We developed an ultrasonic levitation washer that generates silver ions (Ag^+) and ozone (O_3) to clean and sterilize medical devices. We report the effect of ultrasonic levitation (levitation) with Ag^+ and O_3 on MRSA *in vitro* and *in vivo*.

Methods: Antimicrobial effect against 6 MRSA strains of all *agr* types was examined under 3 *in vitro* conditions; cells floating in a water tank, cells infiltrating-, and cells forming a biofilm on an atelocollagen membrane. In the *in vivo* studies we assayed the number of MRSA organisms that survived treatment on murine skin ulcers and evaluated the ulcer size.

Results: Levitation with Ag^+ dramatically decreased the survival of MRSA floating in a water tank. Levitation with Ag^+ and O_3 significantly decreased the viability of MRSA that had infiltrated or formed a biofilm on atelocollagen membranes regardless of the level of biofilm production. *In vivo* studies showed that the number of MRSA on mu-

rine skin ulcers was significantly decreased when 15-min treatment was performed for 7 consecutive days and that the ulcer size was significantly decreased after the 7th treatment course.

Conclusions: Levitation with Ag^{+} and O_3 may be a valuable tool for treating MRSA infestation of the skin and for accelerating wound healing.

Key Words: MRSA, ultrasonic levitation, silver ion, ozone, Infection control,

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA), a major pathogen in nosocomial infections, produces a wide variety of infections ranging from superficial skin/wound- to life-threatening infections such as septicemia. The pathogenesis of *S. aureus* infection is thought to be coordinated by the accessory gene regulator (*agr*) system [1] and *agr* regulates the synthesis of many virulence factors during bacterial growth. [2] *S. aureus* strains are divided into *agr-1, -2, -3, and -4* types; [3,4] *agr-2* is associated with biofilm overproduction. [1,5,6] MRSA grows and forms a biofilm not only on skin and wounds but also on indwelling medical devices. [7,8] According to criteria of the International Standards Organization, clinical instruments must undergo 3 processes to prevent infections, i.e. cleansing, disinfection, and sterilization. An ultrasonic levitation washer disinfector featuring silver electrolysis and ozone oxidation (international patent No. WO 2006/001293 A1) has been developed for cleaning, disinfection and sterilization; [9] it simultaneously generates ultrasonic levitation that has cleansing functions and silver ion (Ag^{+}) and ozone (O_3) that have disinfection and sterilization properties. Use of this device resulted in the killing of *Geobacillus stearothermophilus* and *Bacillus*

atrophaeus, the bacilli most resistant to autoclaving and gas sterilization. [9, 10] Here we first report the antimicrobial effects of levitation with Ag^+ and O_3 on MRSA floating in water, infiltrating atelocollagen membranes, and forming a biofilm on membranes. We examined MRSA of all *agr* types with different biofilm-forming abilities. Using murine skin ulcers and an *agr-2* MRSA strain, we also investigated the antimicrobial and wound healing effects of this device.

Methods

Ultrasonic levitation washer with silver electrolysis and ozone oxidation

The device consists of a water tank, ultrasonic wave transducers, driving circuits, an ozone generator, and a silver electrolytic sterilization system (Fig. 1). The 150 x 150 x 295 mm water tank is made of stainless steel; the water volume we used was 4.5 liters. Two umbrella-shaped, independently-driven ultrasonic transducers are at the bottom of the water tank. Ultrasonic vibrations (25 kHz), generated by piezoelectric ceramic elements, are comprised of radial progressive waves emitted from independent

sources; the waves are superimposed on each other. The shearing impact effect for aqueous cleaning is obtained by interference from vertical and horizontal waves. This mode of ultrasonic wave generation, designated "levitation" by Shigihara and Ueda (T. Shigihara, T. Ueda, 22 June 2005, International Patent Office) yields advantages including uniformity of washing efficacy, even distribution of intensity, and wide applicability. [9, 10] Ozone-containing air is conducted to the water tank through silicone tubes and introduced into the water via 4 holes on each oscillator (Fig. 1a). Each function of the ozone oxidation and silver electrolysis can be used individually or in combination. Silver ions are eluted into the water from a silver electrode plate mounted on the lid (Fig. 1b). Direct-current (DC) voltage is then applied between the silver electrode plate (anode) and the water tank (cathode). The concentration of silver ions dissolved in water was measured on an atomic absorption photometer (AAnalyst800, PerkinElmer Japan Co., Ltd. Kanagawa, Japan). As shown in Table 1, the concentration of silver ions increases time-dependently. We used a 900-ml water tank with one oscillator and silver electrolysis to study floating cells.

MRSA strains

MRSA strains were isolated from skin exudates of patients at Kagoshima University Hospital (Table 2). Culture specimens were collected as usual for routine clinical cultures. Specimens were incubated on 5% sheep blood agar at 37°C for 24 hr. *S. aureus* was identified by colony morphology and a coagulase test. Resistance to methicillin was determined by subculturing isolates on MRSA screening plates (Becton Dickinson, Franklin Lakes, NJ) at 35°C for 24 hr. The strains were individually stored at 80°C in 10% skim milk.

Genotyping of *agr* and quantitative test of biofilm

Using the multiplex polymerase chain reaction assay, 4 *agr* types were identified. [11] Quantitative tests with the microtiter plate assay [12] confirmed that all strains formed a biofilm. Briefly, after overnight incubation of the strains in trypticase soy broth (TSB) 5 μl of the suspension were inoculated into 200 μl of TSB containing 0.25% glucose in 96-well, flat-bottom, polystyrene microtiter plates. This was followed by 18-hr incubation at 37°C without shaking. All experiments were carried out in du-

plicate. Adherent cells and the matrix at the bottom of the wells were recognized as biofilm. Then the supernatant was discarded, the wells were washed gently 4 times with tap water to remove cells deposited on the bottom, the biofilm was stained with 0.1% crystal violet for 5 min, the staining solution was discarded, and the wells were again washed 4 times. The stained biofilm was resolved in 200 μl of 95% ethanol. Optical density at 595 nm (OD_{595}), regarded as the biofilm index, was measured with an ELISA plate reader (Model 550, Japan Bio-Rad Laboratories, Tokyo, Japan). Strains with OD_{595} exceeding 0.304 were defined as biofilm-overproducing. [6]

Effect of levitation with Ag^+ on MRSA floating in the water tank

A cell suspension of the *agr-2* MRSA strain (strain A), spectrophotometrically adjusted to the turbidity of a MacFarland 0.5 scale, was poured into the water tank. During the 10-min levitation treatment with Ag^+, 1-ml samples were collected at 2-min intervals from the surface and the middle of the water tank. The samples were diluted, plated on standard agar plates (Pearlcore® nutrient agar, EIKEN Chemical Co. Ltd.,

Tochigi, Japan), and incubated at 37°C for 24 hr. Mean colony-forming units (cfu)/ml were counted by triplicate counts.

Effect of levitation with or without Ag^+ or O_3 on MRSA infiltrating the atelocollagen membrane

A cell suspension of 6 MRSA strains that included all *agr* types were adjusted spectrophotometrically to the turbidity of a MacFarland 0.5 scale, and diluted 1000 times with phosphate buffered saline (PBS). 20 µl of diluted solution were inoculated with a micropipette onto a 1 x 1 cm atelocollagen membrane (Pelnac®; Gunze Co. Ltd., Kyoto, Japan). Samples were treated by levitation with or without Ag^+ or O_3 for 15 min. Control experiments were performed under identical conditions but without levitation, Ag^+, or O_3. After treatment, the membrane was dissolved in 200 µl of collagenase (from *Clostridium histolyticum*; Sigma-Aldrich Inc., St. Louis, MO) for MRSA extraction. The extracts were diluted, plated on standard agar plates, and incubated at 37°C for 24 hr. Mean cfu/ml values were determined by triplicate counts. The relative viabil-

ity index was defined as the ratio of cfu/ml after 15-min treatment to the ratio in the control.

Effect of levitation with or without Ag^+ or O_3 on MRSA forming a biofilm on the atelocollagen membrane

Biofilm formation on the atelocollagen membrane was induced with a modification of the microtiter plate assay. [12] Briefly, strains were grown overnight in TSB, 15 μl of the suspension were inoculated into 3 ml of TSB containing 0.25% glucose in 12-well, flat-bottom, polystyrene microtiter plates containing a 4 cm^2 piece of atelocollagen membrane, and the plates were then incubated at 37°C for 18 hr without shaking. The supernatant was discarded, the membrane was washed gently 3 times, dried gently, treated by levitation with or without Ag^+ or O_3 in the water tank, and a 1-cm^2 membrane piece was dissolved in 200 μl of collagenase. The extracts were diluted, spread on standard agar plates, and incubated at 37°C for 24 hr. Mean $\log_{10}$ cfu/sample values were determined by triplicate counts. Extracts from the atelocollagen membranes were stained using the *Bac*Light™ bacterial viability kit (Invitrogen, Paisley, UK). Viable

and dead MRSA, stained green with Syto-9™ (S-9) and red with propidium iodide (PI), respectively, were visualized under a fluorescence microscope (Bz-8000; Keyence, Osaka, Japan).

Animals

Male C57BL/6 mice (6/group) weighing from 20 to 25 g (CLEA Japan Inc., Tokyo, Japan) were used. They were housed in individual cages under constant temperature (28°C) and humidity (55 ± 10%) at a 12 hr light/dark cycle; food and water were *ad libitum* throughout the study. All procedures were approved by the Institute of Laboratory Animal Sciences, Kagoshima University (Frontier Science Research Center).

***In vivo* effect of levitation with Ag^+ and O_3 on *agr*-2 MRSA inoculated onto murine skin ulcers**

The mice were anesthetized with 100 mg/kg ketamine and 10 mg/kg xylazine and made 2 ulcers on their back with a 5-mm dermatological biopsy punch. A cell suspension of MRSA (strain A) was adjusted spectrophotometrically to the turbidity of a

MacFarland 0.5 scale and diluted 10,000 times with PBS. A volume of 5 μl of suspension and 5 μl of TSB containing 0.25% glucose were inoculated with a micropipette onto each ulcer. The ulcers were covered with polyurethane dressing (Hydrosite thin type®, Smith & Nephew Wound Management Inc., Hull, UK) for 3 days. Then the mice were fixed on a corkboard and their ulcer-bearing back, immersed in the water tank, was treated by levitation with Ag^+ and O_3 for 15 min/day for 7 days. Control mice were immersed in water. On the last day of treatment, the mice were sacrificed and a 1.0-cm^2 piece of skin that included the ulcers was surgically removed and homogenized in 1 ml of PBS. Diluted supernatant was plated on MRSA-selective agar (Becton Dickinson, Franklin Lakes, NJ) and mean $\log_{10}$ cfu/cm^2 was determined in triplicate. The longest (L) and shortest (W) axes were measured and the ulcer area (A) was calculated as $(A) = \pi (L \times W) / 4$.

Statistical analysis

Student's *t*-test, the Tukey- and the Dunnett test were performed using statistical analysis software (Dr. SPSS II for Windows; SPSS Inc., Tokyo, Japan). Differences of $p < 0.05$ were regarded as statistically significant.

Results

Effect of levitation with Ag^+ on MRSA floating in the water tank

The survival of MRSA (strain A) in the tank was significantly reduced by 2-min levitation treatment with Ag^+ (Fig. 2). Fewer than 1 cfu of MRSA per ml survived after 10-min treatment. As in earlier studies, [2] the intensity of levitation was weaker on the water surface than inside the tank. However, the antimicrobial effect was not significantly different.

Effect of levitation with or without Ag^+ or O_3 on MRSA infiltrating the atelocollagen membrane

Atelocollagen membranes infiltrated by MRSA were prepared as in our *in vitro* model of MRSA contamination of wound tissue. The number of *agr-2* MRSA (strain A) was significantly reduced by 10-min levitation treatment with Ag^+ or O_3, or both (Fig. 3a). After 5-min treatment, the presence of Ag^+ and O_3 had an additive effect on the decreased MRSA count. The number of MRSA organisms was significantly decreased by 15-min levitation treatment with Ag^+ and O_3 regardless of the *agr* type of the other 5 strains (Fig. 3b).

Effect of levitation with or without Ag^+ or O_3 on MRSA forming a biofilm on the atelocollagen membrane

Levitation with or without Ag^+ or O_3 significantly reduced the number of biofilm-forming *agr-2* MRSA (strain A) in a time-dependent manner (Fig. 4a). Levitation, Ag^+, and O_3 had a synergistic effect after 15-min treatment. The number of biofilm-forming MRSA organisms on the membrane was also significantly reduced by 30-min levitation with Ag^+ and O_3 regardless of the *agr* type of the other 5 strains (Fig. 4b). Examination under a fluorescence microscope revealed a time-dependent decrease in

the number of viable cells and an increase in dead cells upon levitation with Ag^+ and O_3 (Fig. 5).

In vivo effect of levitation with Ag^+ and O_3 on _agr-2_ MRSA inoculated onto murine skin ulcers

We investigated the *in vivo* effect of levitation with Ag^+ and O_3. The number of MRSA (strain A) on the murine ulcers was significantly decreased by daily levitation treatment with Ag^+ and O_3 (15 min/day for 7 days) (Fig. 6a). The size of MRSA-colonized ulcers was also significantly diminished on day 7 (Fig. 6b). The body weight loss was less than 10% in all treated mice (data not shown).

Discussion

As MRSA infection of the skin or soft tissue may necessitate postponement of surgery, resulting in the prolongation of hospitalization and increased medical costs, innovative simple and effective antimicrobial modalities are needed to reduce MRSA col-

onization and prevent MRSA infection. The ultrasonic levitation washer combined with Ag^+ and O_3 was developed to clean and sterilize instrumentation. [9] Previous studies showed that the device killed *Geobacillus stearothermophilus* and *Bacillus atrophaeus*, organisms that were highly resistant to autoclaving and gas sterilization. [9, 10] When we investigated the effect of levitation combined with Ag^+ and O_3 on MRSA, we found that levitation with Ag^+ dramatically decreased the viability of MRSA floating in water and that levitation with Ag^+ or O_3, or both, decreased the viability of MRSA of all *agr* types. Consistent with earlier studies showing that levitation alone did not kill *Escherichia coli* [9] we found that 15-min levitation alone had no effect on MRSA infiltrating atelocollagen membranes. While levitation effectively cleanses instrumentation, it exerts no antimicrobial effects. On the other hand, Ag^+ and O_3 exhibit a broad antimicrobial spectrum. [13-15] Silver ions bind to biological molecules containing thio-, amino-, carboxylate-, imidazole-, or phosphate groups in the cell wall and cell membrane, and in enzymes and DNA, resulting in bacterial inactivation and destruction. [16, 17] Ozone directly oxidizes and destroys the bacterial cell wall, cell membrane, and nucleic acids, leading to cytolysis. [18, 19] We found that treatment with Ag^+ or O_3, or both, without levi-

tation had weaker antimicrobial effects than did treatments that also included levitation (data not shown). Our findings suggest that levitation weakens the attachment of MRSA to the atelocollagen membrane and that Ag^+ and O_3 then efficiently kill the MRSA organisms. Although an earlier report suggested that microbial wound contamination might not persist, flourishing species have been shown to establish states described as colonization, critical colonization, and wound infection. [20, 21] We posited that MRSA infiltration into atelocollagen membranes represents an *in vitro* model of contamination and obtained data suggesting that levitation with Ag^+ and O_3 may combat surgical site contamination. Biofilm plays an important role in the pathogenesis of *S. aureus* infections; it complicates the eradication of biofilm-associated infections and may lead to persistent and chronic diseases attributable to this organism. [22-24] Our biofilm formation assay showed remarkable killing of MRSA upon levitation with Ag^+ and O_3 regardless of the *agr* type or the biofilm index. In addition, 30-min levitation tended to decrease the viability of MRSA in biofilm. These observations suggest that levitation affects biofilm stability and that Ag^+ and O_3 directly diminish the viability of MRSA. We document that levitation with Ag^+ and O_3 exerted antimicrobial effects in our

MRSA colonization model of murine skin ulcers and that the size of MRSA-colonized ulcers was markedly decreased. While we did not examine in detail the effect of levitation alone on murine skin ulcers, it may support wound healing because others have shown that low-frequency ultrasound therapy clinically accelerated wound healing. [25-28]

In our study, influence of levitation with Ag^+ and O_3 on human skin cells was not examined. Over the past few decades, many therapeutic instruments using silver and ozone have been developed.[29, 30] Despite various therapies with ozone for infectious disease of the skin, oral mucosa and vagina are reported, [29, 31] there is no adverse effect to human skin. Although ozone is known to have toxic pulmonary effect, [32] the concentration of O_3 gas at 30 cm above the device (data not shown) is within the permissible range set by labor and environmental standards, indicating that the use of this device is safe for humans. While the possible adverse effect of Ag^+ is allergic and irritant contact dermatitis and sensitization, [30, 33-35] ion concentration is much less compared to direct contact with silver. Although further studies are needed to confirm its effectiveness and safety in humans, our findings suggest that levitation with Ag^+ and O_3 is a valuable tool for treating MRSA infestation of the skin and for preventing surgical

site infection. The device used in this study was designed for disinfecting human hands and feet. A bathtub-type device to treat infected wounds on the whole body has been developed. We disinfect the tank between patients by 60-min treatment with sodium hypochlorite solution (0.001-0.002%), an amphoteric surface active agent, or quaternary ammonium salts (0.2-0.5w/v%), or by wiping with alcohol. This treatment may also be applicable to the disinfection of the hands of care providers and may help to eradicate nosocomial infections. It may prevent the delay of surgery that results in prolonged hospitalization, the outbreak of other drug-resistant strains, and consequent medical cost increases.

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Table 1. Concentration of Ag^+ dissolved in water (mean ± SD)

Time (min)	Ag^+ concentration (mg/l)
5	0.103 ± 0.002
10	0.108 ± 0.002
20	0.127 ± 0.001
30	0.151 ± 0.001

Ag^+ was increased in a time-dependent manner.

Table 2. MRSA strains

Strain	Infection/Carrier	Underlying disease	Biofilm index (OD_{595})	*agr* type
A	Infection	Darier's disease	0.742	2
B	Infection	Impetigo	1.855	3
C	Infection	Postoperative wound	0.753	2
D	Infection	Toxic epidermal necrolysis	0.525	4
E	Carrier	Atopic dermatitis	0.397	1
F	Carrier	Malignant melanoma	0.518	1

MRSA strains (n=6) were isolated from skin exudates of patients with the indicated diseases and the biofilm index and *agr* type were analyzed. All strains were biofilm-overproducing; their OD_{595} exceeded 0.304.

Figure Legends

Figure 1. Schematic presentation of the ultrasonic levitation washer disinfector with silver electrolysis and ozone oxidation.

The device consists of a water tank, ultrasonic wave transducers, driving circuits, an ozone generator (a) and a silver electrolysis sterilization system (b). Two umbrella-shaped ultrasonic transducers at the bottom of the tank generate ultrasonic waves ("levitation"). Ultrasonic levitation, silver ions, and ozone can be generated separately or simultaneously. The scheme is presented with permission from a previous report (10).

Figure 2. The effect of levitation and Ag^+ on MRSA floating in the water tank.

During the 10-min levitation treatment with Ag^+, samples were collected at 2-min intervals from the surface and the middle of the water tank. The survival of MRSA (strain A) was significantly reduced after 2- min treatment irrespective of the water depth. The data shown represent mean ± SD of triplicate determinations of one representative experiment of 3 separate experiments. (* $p < 0.05$, NS = not significant)

Figure 3

(a). The effect of levitation with or without Ag^+ or O_3 on MRSA infiltrated in the atelocollagen membrane.

Equal amounts of MRSA (strain A) were inoculated onto a 1 x 1 cm atelocollagen membrane. Samples that represent an in vitro model of contamination were treated for 15 min. The number of MRSA was significantly reduced by 10-min levitation with Ag^+ or O_3, or both. After 5 min, Ag^+ and O_3 had an additive effect. (a *vs* e, a *vs* f, a *vs* g: $p < 0.001$; b *vs* c, c *vs* d, b *vs* d: $p < 0.05$.)

(b). Relative viability index of various *agr* types of MRSA infiltrated in the atelocollagen membrane.

Samples that a 1 x 1 cm atelocollagen membrane inoculated equal amounts of 6 MRSA strains were treated for 15 min. The relative viability index was defined as the ratio of cfu/ml after 15-min treatment to the ratio in the control. The num-

ber of MRSA was significantly decreased by 15-min levitation with Ag^+ and O_3 regardless of the *agr* type. (*$p < 0.001$, compared with the control)

The data shown represent mean ± SD of triplicate determinations of one representative experiment of 3 separate experiments.

Figure 4

(a). The effect of levitation with or without Ag^+ or O_3 on *agr-2* MRSA forming a biofilm on the atelocollagen membrane.

Samples that formed biofilm on the atelocollagen membrane were treated for 60 min. The number of MRSA (strain A) was significantly reduced by levitation with Ag^+ and O_3. A synergistic effect of levitation, Ag^+, and O_3 was observed after 15 min. (a *vs* b, a *vs* c, a *vs* d, a *vs* e, $p < 0.001$.)

(b). The effect of levitation with Ag^+ and O_3 on MRSA forming a biofilm on the atelocollagen membrane.

Samples that formed biofilm of 6 MRSA strains on the atelocollagen membrane were treated for 30 min. The number of MRSA was significantly decreased by

levitation with Ag^+ and O_3 regardless of the *agr* type or the biofilm index. (*p < 0.001, **p < 0.05, compared with the control)

The data shown represent mean ± SD of triplicate determinations of one representative experiment of 3 separate experiments.

Figure 5. Fluorescence microscopic examination of *agr-2* MRSA.

After treatment with levitation, Ag^+ and O_3, extracts from the atelocollagen membranes were stained using the *Bac*Light™ bacterial viability kit. Viable and dead MRSA, stained green with Syto-9™ (S-9) and red with propidium iodide (PI), respectively, were visualized under a fluorescence microscope. Bar = 50 μm. Data shown is the representative one of 3 separate experiments.

(a). In the control study, experiments were performed under identical conditions but without levitation, Ag^+, or O_3. There was no increase in dead- and no decrease in viable cells.

(b). Levitation with Ag^+ and O_3 reduced the number of viable cells and increased the number of dead cells in a time-dependent manner.

Figure 6

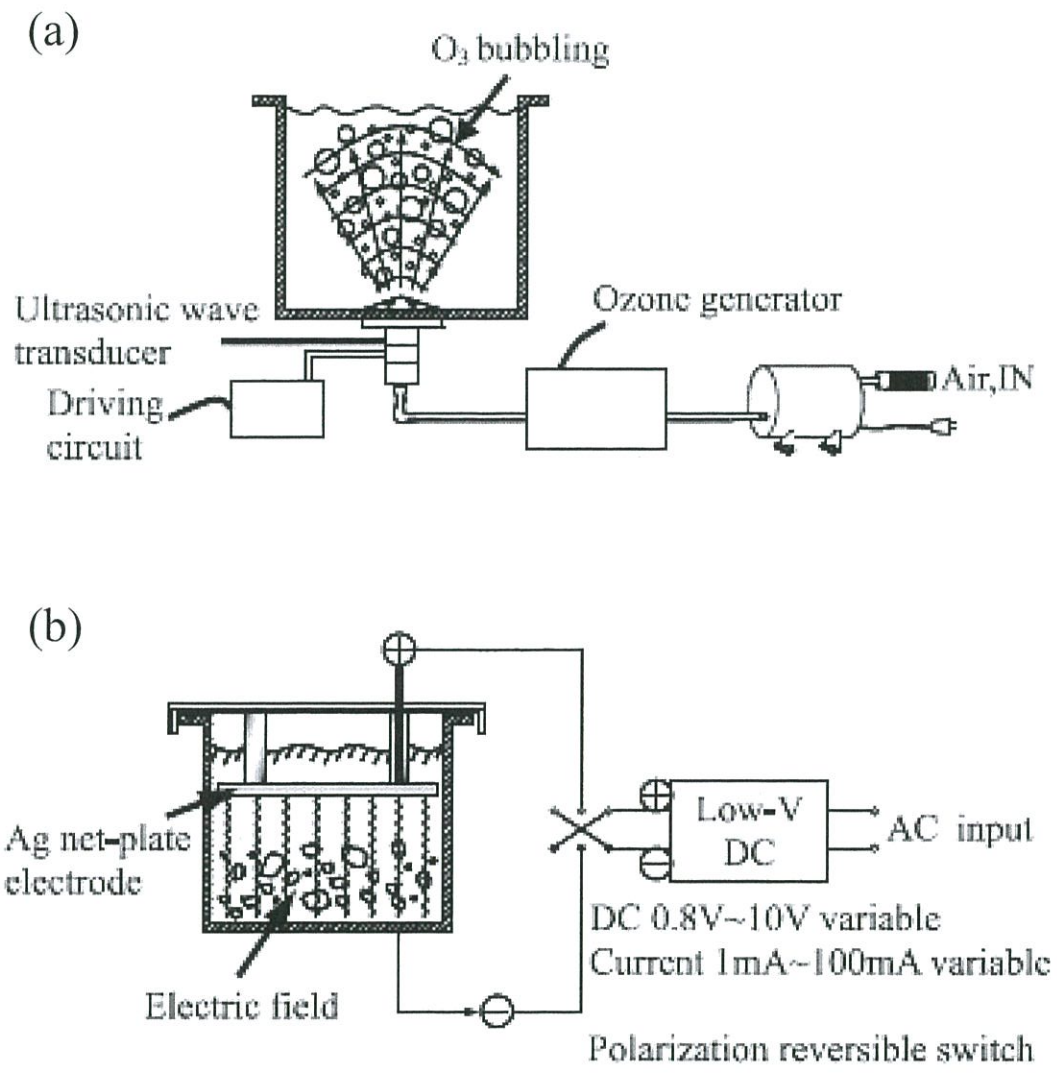
(a). *In vivo* effect of levitation with Ag^+ and O_3 on *agr-2* MRSA colonization model onto murine skin ulcers.

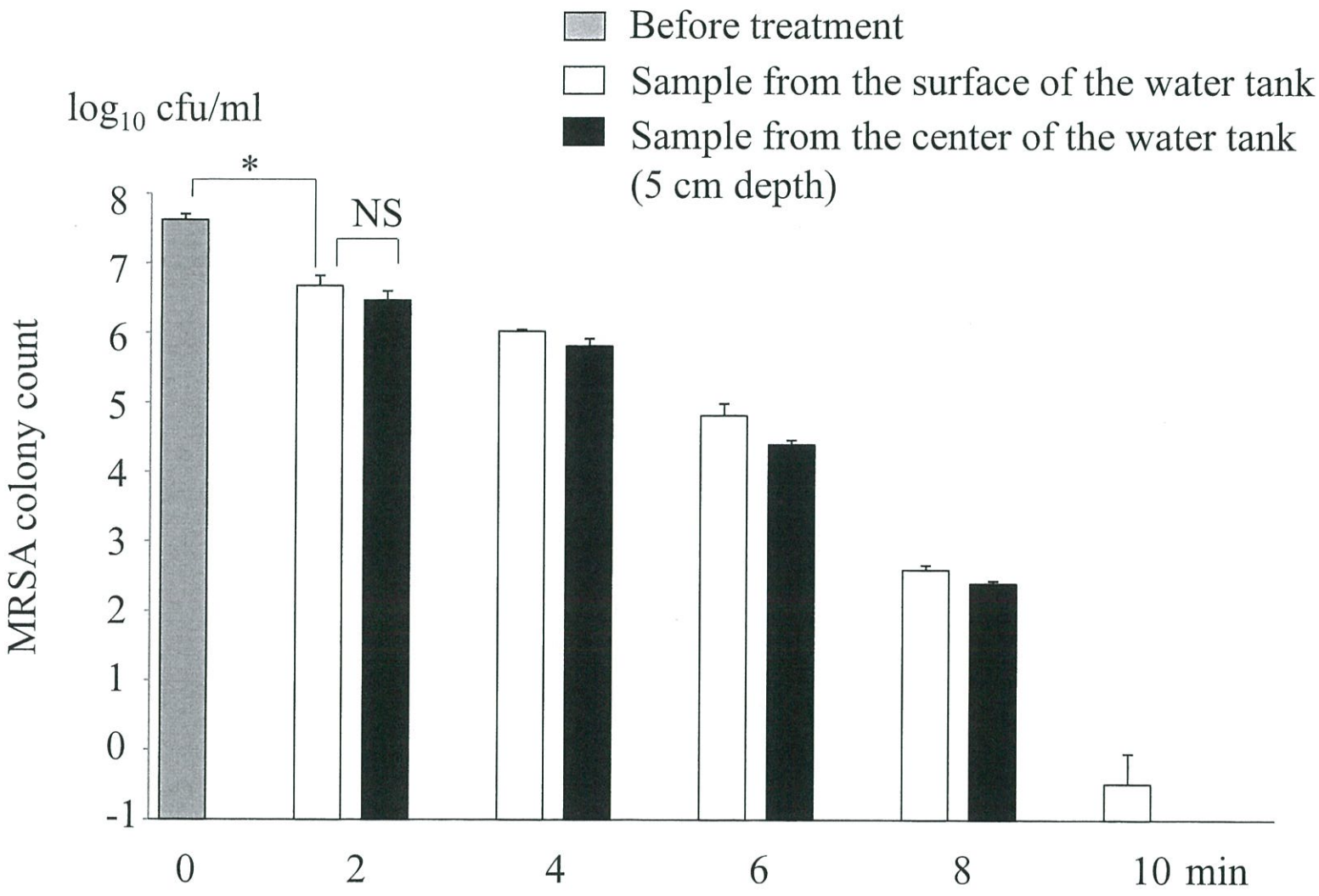
Murine skin ulcers that colonized equal amounts of MRSA (strain A) were treated by levitation with Ag^+ and O_3 for 15 min/day for 7 days. The number of MRSA was significantly lower after the 7th levitation course with Ag^+ and O_3 compared to the control. (*$p < 0.001$, NS = not significant)

(b). The effect of levitation with Ag^+ and O_3 on MRSA-colonized murine skin ulcers.

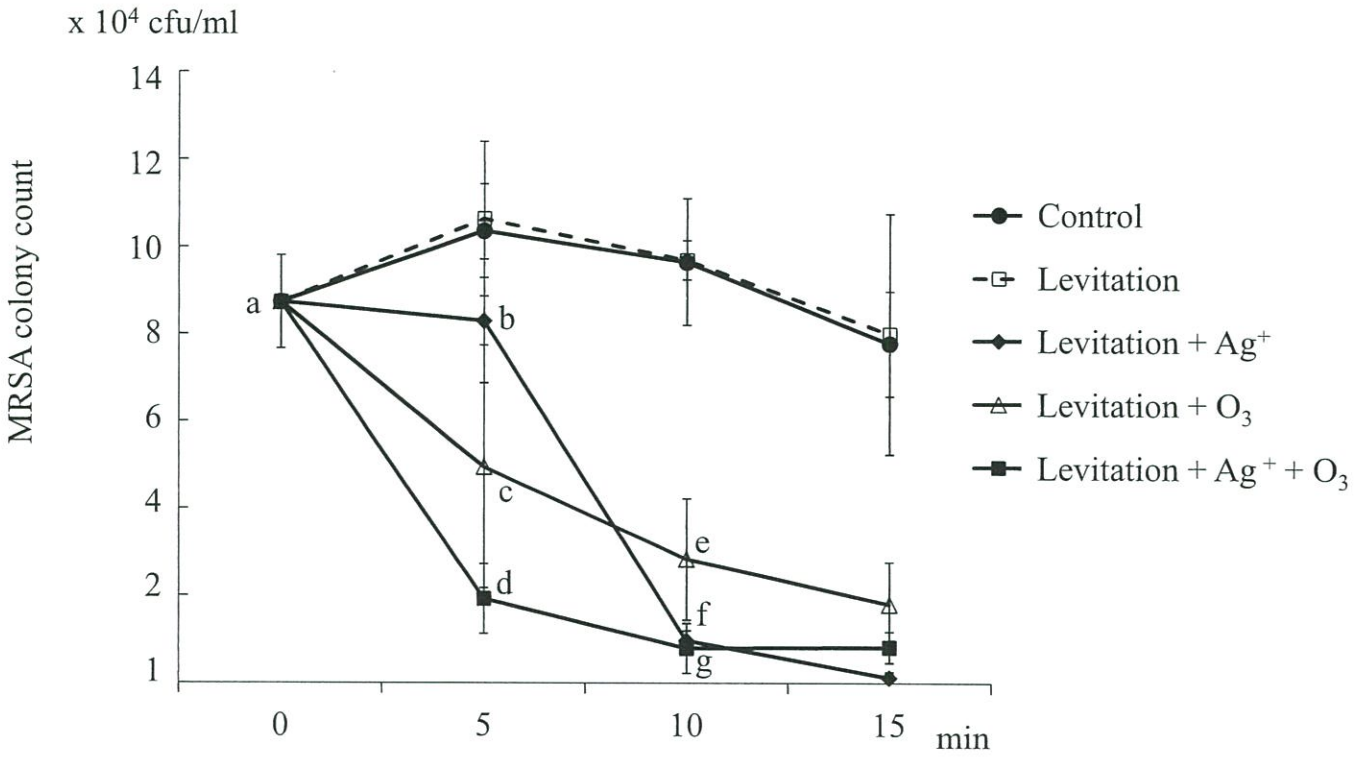
The ulcer area was measured before treatment and after the 7th treatment. The size of MRSA-colonized ulcers was significantly smaller after the 7th levitation course with Ag^+ and O_3 compared to the control. (*$p < 0.001$, NS = not significant)

The data shown represent mean ± SD of 12 determinations of one representative experiment of 2 separate experiments.

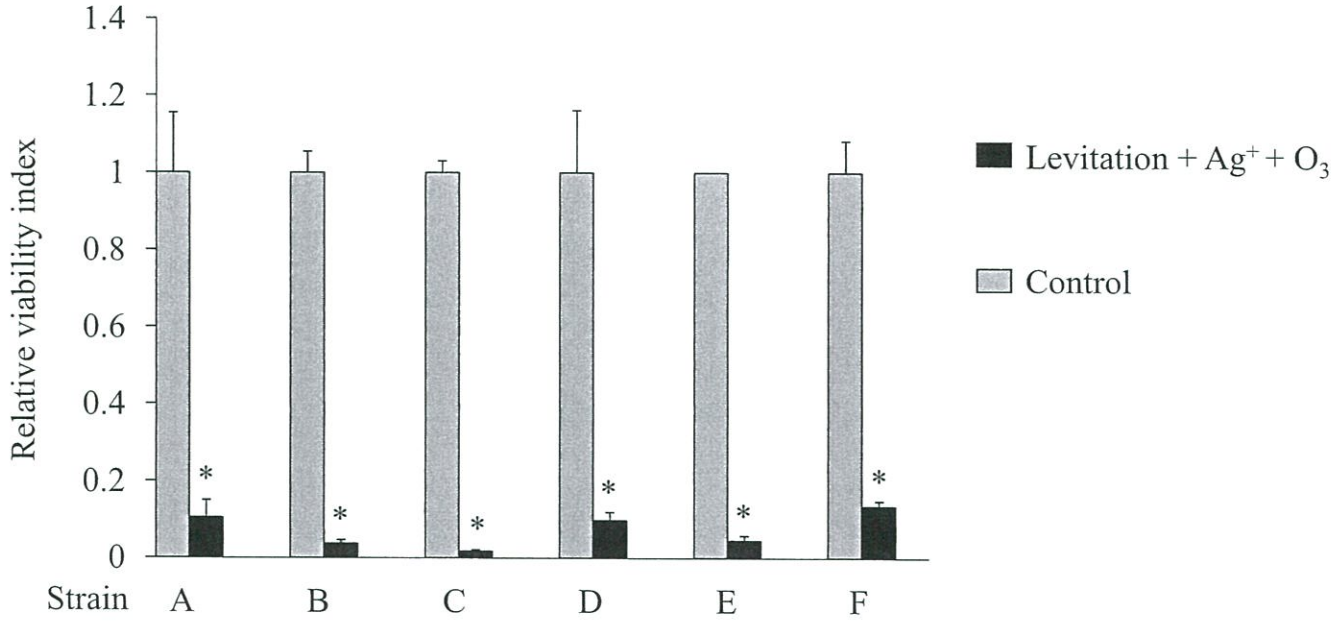

(a)
O_3 bubbling
Ultrasonic wave transducer
Driving circuit
Ozone generator
Air,IN
(b)
Ag net-plate electrode
Electric field
Low-V DC
AC input
DC 0.8V~10V variable
Current 1mA~100mA variable
Polarization reversible switch


Before treatment
Sample from the surface of the water tank
Sample from the center of the water tank
(5 cm depth)
log10 cfu/ml
MRSA colony count
*
NS
8
7
6
5
4
3
2
1
0
-1
0
2
4
6
8
10 min

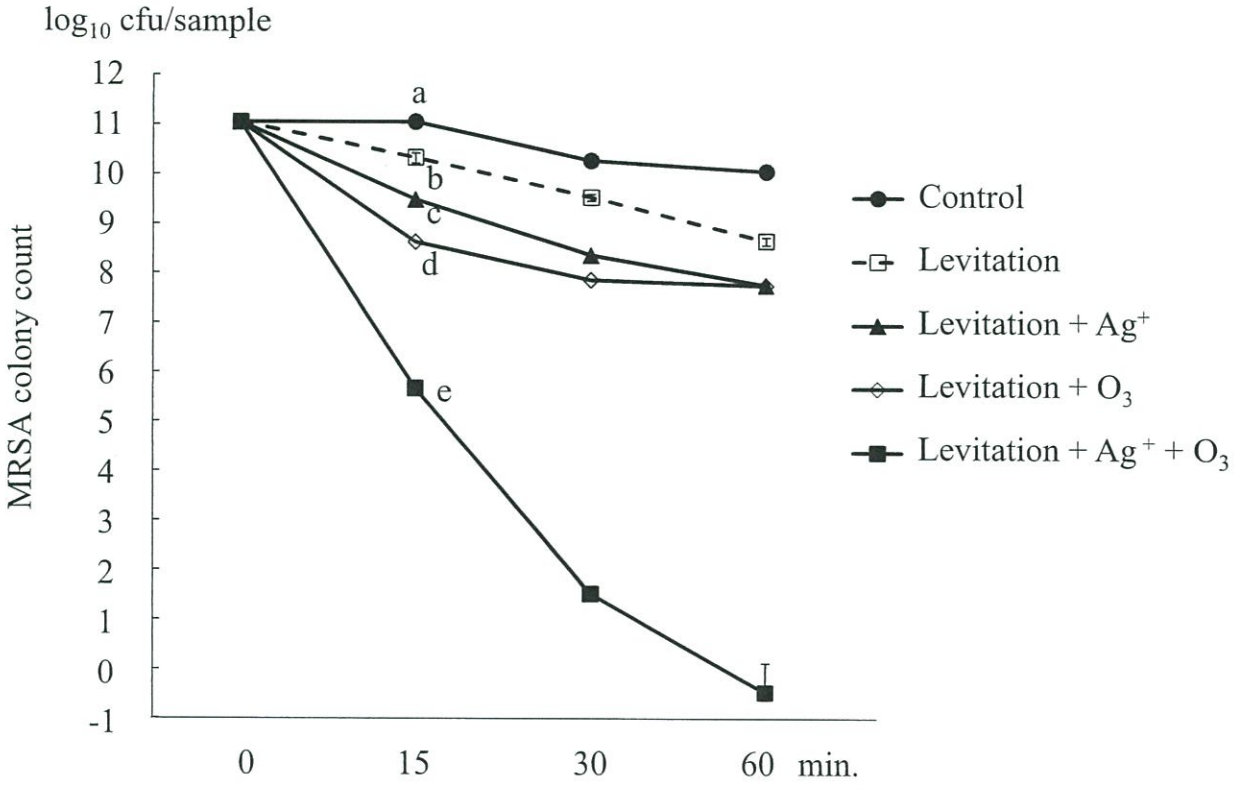
(a)


x 10⁴ cfu/ml
MRSA colony count
14
12
10
8
6
4
2
1
0
5
10
15
min
a
b
c
d
e
f
g
Control
Levitation
Levitation + Ag+
Levitation + O3
Levitation + Ag+ + O3

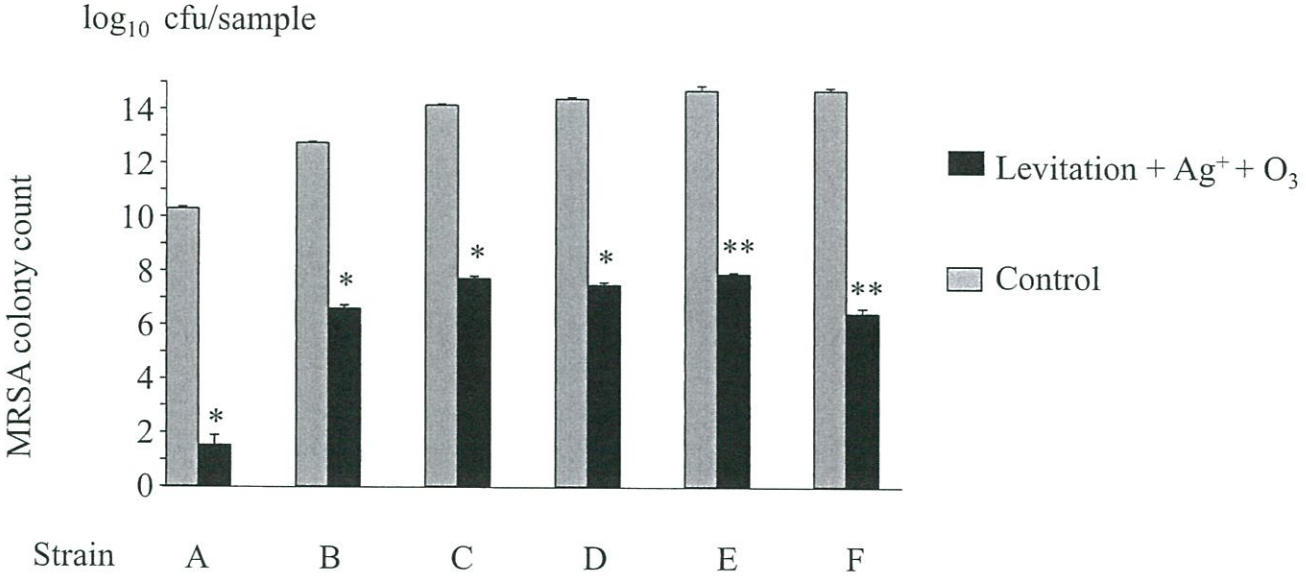
(b)


Relative viability index
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Strain
A
B
C
D
E
F
*
Levitation + Ag+ + O3
Control

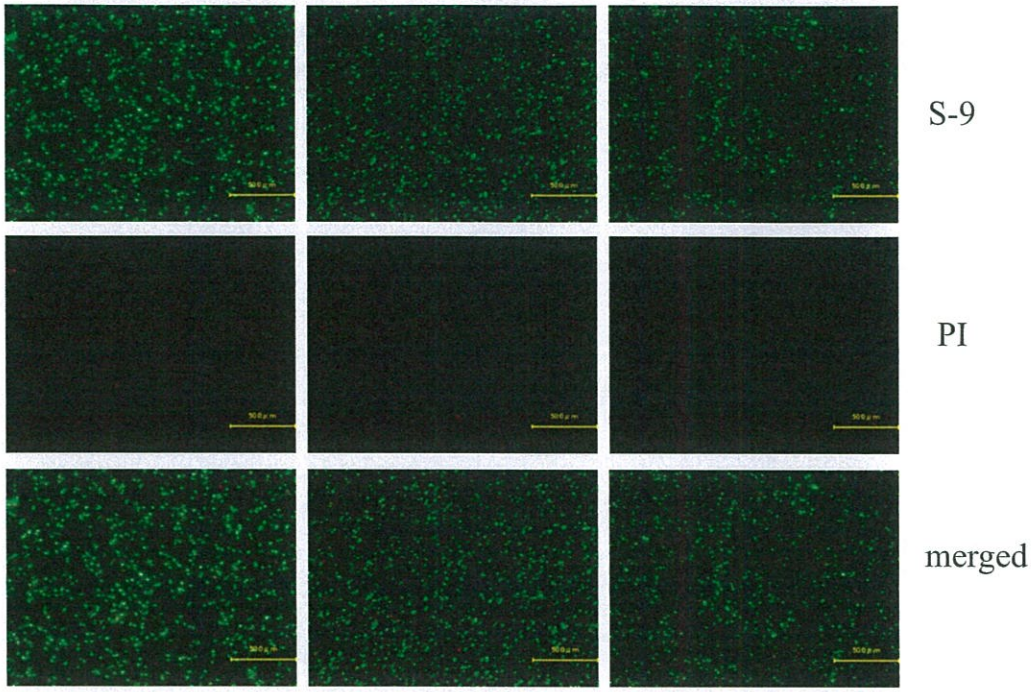
(a)


log10 cfu/sample
MRSA colony count
12
11
10
9
8
7
6
5
4
3
2
1
0
-1
a
b
c
d
e
0
15
30
60 min.
Control
Levitation
Levitation + Ag+
Levitation + O3
Levitation + Ag+ + O3

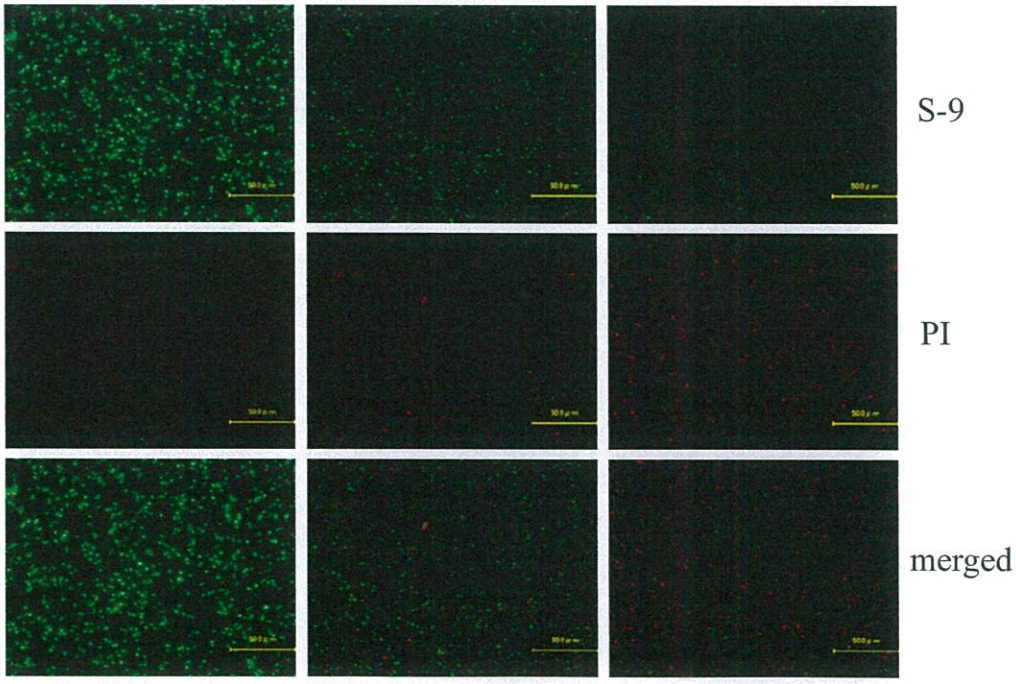
(b)


log10 cfu/sample
MRSA colony count
14
12
10
8
6
4
2
0
*
*
*
*
**
**
Strain
A
B
C
D
E
F
Levitation + Ag+ + O3
Control

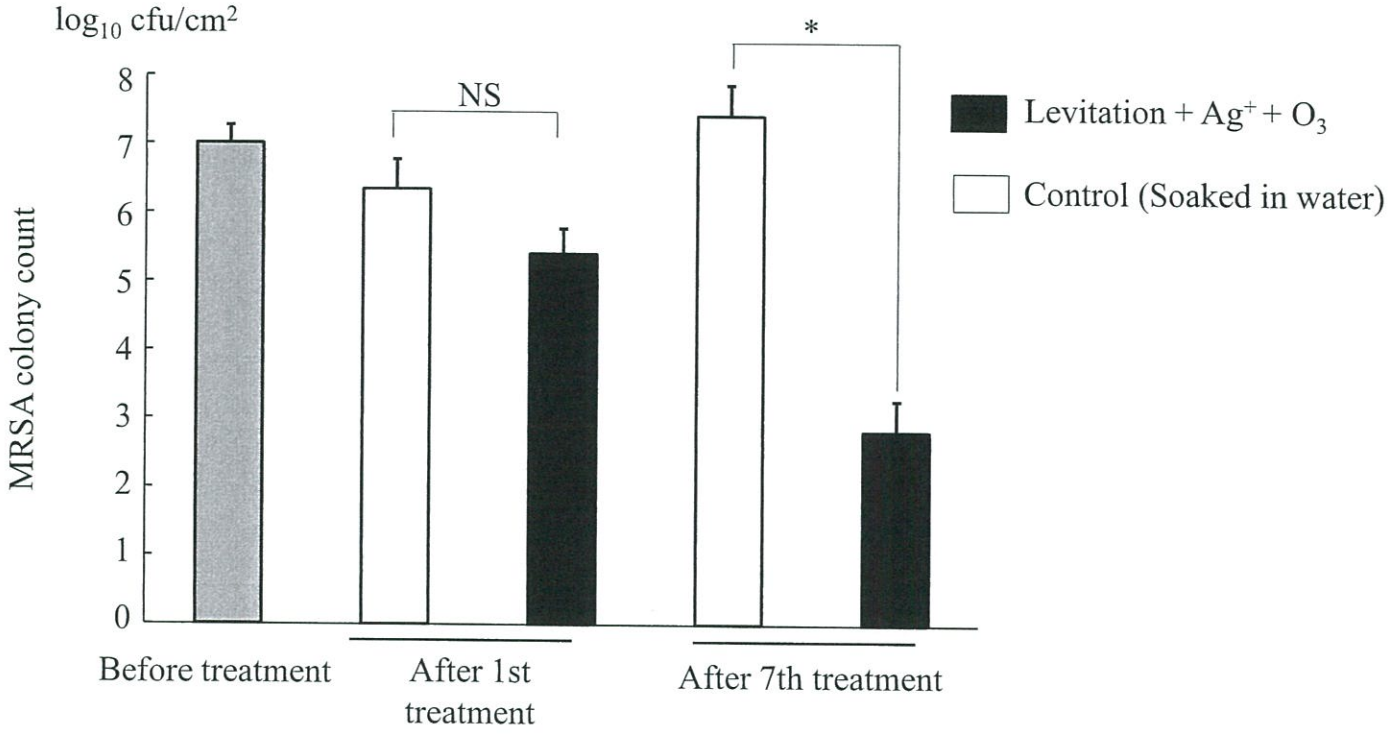
(a)



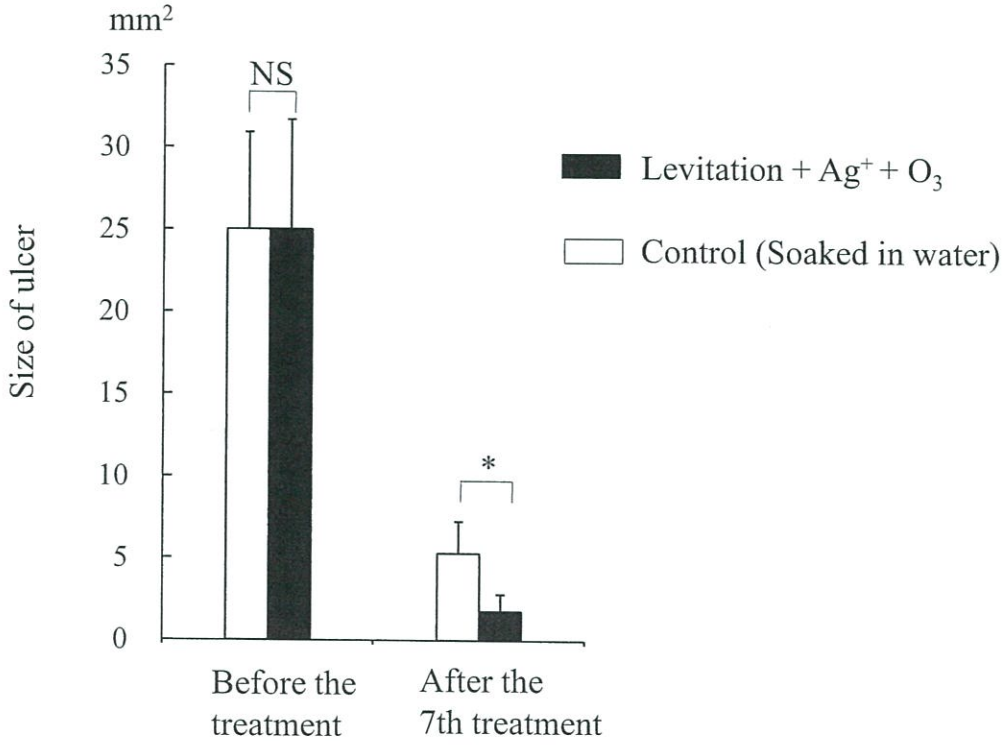
(b)



(a)


log10 cfu/cm2
MRSA colony count
8
7
6
5
4
3
2
1
0
NS
*
Levitation + Ag+ + O3
Control (Soaked in water)
Before treatment
After 1st treatment
After 7th treatment

(b)


mm2
Size of ulcer
35
30
25
20
15
10
5
0
NS
*
Levitation + Ag+ + O3
Control (Soaked in water)
Before the treatment
After the 7th treatment