Effectiveness of acid-electrolyzed functional water for oral care of the elderly: an in vitro study

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Abstract

Background: Aspiration pneumonia is a major cause of death in the elderly. Oral bacterial can contribute to the occurrence of this disease. It is therefore important to maintain oral cavity hygiene. However, tooth brushing is sometime difficult for elderly people. Acid-electrolyzed functional water (FW) is an efficient bactericide, and gargling with FW might therefore contribute to the effective prevention of aspiration pneumonia. We investigated the possible use of FW as a mouth rinse.

Methods: The bactericidal effect of FW against each species of bacteria was evaluated using the numbers of colony-forming units. The test organisms were *Staphylococcus aureus*, *Streptococcus pneumonia, Pseudomonas aeruginosa,* and *Candida albicans.* The experiment was conducted using PBS as a control, LISTERINE, and ConCool F. We used two concentrations of ConCool F: undiluted, and the optimal concentration indicated by the manufacturer.

To investigate the bactericidal mechanism of FW, the activity of superoxide dismutase, an indicator of oxidative action, was measured in *S. aureus*. FW was diluted with purified water to concentrations of 10, 30, 50, and 70%. The number of colony-forming units were measured for each concentration. XTT assays were performed using HSC-3 and HeLa cells, to examine the viability of the cells following treatment with FW. The same experiment was conducted with PBS, Listerine, and undiluted ConCool F.

Results: No bacteria treated with FW, Listerine, or undiluted ConCool F formed colonies. However, the number of colony-forming units in bacteria treated with diluted ConCool F was equivalent to that of control, except for *C. albicans.* Superoxide dismutase activity peaked at a 50% concentration of FW, and was more than twice that of the control. A significant decrease in the number of colony-forming units was observed following 50% treatment. Since the peaks of the superoxide dismutase activity and the starting concentrations of the bactericidal effects coincided, the bactericidal effect of FW's might be related to their oxidative effects. Bacteria treated with FW had as higher a survival rate than the other two mouth rinses.

Conclusions: Our results suggest that FW might be clinically applicable as a mouth rinse.

Background

The average life expectancy continues to increase worldwide [1]. However, healthy life expectancy is generally more important than lifespan itself for the elderly. Healthy life expectancy is the period in which the elderly do not need significant care, and the gap between healthy life expectancy and average life expectancy is currently a matter of global concern. This gap creates a considerable financial and social burden [2]. The increasing number of elderly individuals, and the length of time for which they need nursing care, have led to increases in medical costs. Extending healthy life expectancy is therefore an urgent issue [3]. Among the various diseases that shorten healthy life expectancy, aspiration pneumonia is particularly relevant to the field of dentistry [4]. Aspiration pneumonia is major concern in the elderly [5].

It is widely recognized that aspiration pneumonia is related to poor oral hygiene, and that oral care is vital for its prevention [6, 7].

There are various approaches to oral care which can prevent aspiration pneumonia, but the central method is tooth brushing by the individual to physically remove bacteria [8]. However, in clinical practice, it is often observed that the oral cavity of the elderly is unhygienic. The main factor is disability. Elderly individuals may not be able to perform fine hand movements, due to functional decline [8]. It is not uncommon for caregivers to brush the teeth of elderly individuals because they cannot do it themselves.

Given this situation, it is important to develop a practical and straightforward method of oral care for the elderly. Gargling is one way to achieve this cleansing, and various methods involving gargling have been proposed. There have been many reports on the effects of chlorhexidine gluconate [9–12]. However, the use of chlorhexidine gluconate carries a risk of anaphylactic shock [13], and the elderly need safer mouth rinses. We focused on using acid-electrolyzed functional water (FW), which is produced by electrolyzing low concentrations of saline, and collecting the product from the anode chambers [14, 15]. FW has been used in a variety of situations in the dental field. However, there have been no reports on its usefulness as a mouth rinse for the elderly. Laboratory evidence has not demonstrated a strong antimicrobial effect of FW against the oral microorganisms which are related to aspiration pneumonia. We hypothesized that FW could be an efficient mouth rinse, and in this study we investigated the bactericidal effects of FW, and its mechanism of action on bacteria associated with the development of aspiration pneumonia. We also investigated the impact of FW on the host cells. This study is a preliminary step toward the practical application of FW for oral care.

Methods

Purification of FW

FW (actual chloride concentration (ACC) 30 ppm, pH 2.2–2.7, oxidation–reduction potential of more than 1,100 mV) was obtained using the Oxilyzer (Mura Denshi, Akita, Japan). The ACC was measured using an ACC measuring kit (Sibata, Soka, Japan). pH values were measured with a pH measuring device (ASONE, Osaka, Japan).

Culture of microorganisms

There is are many microorganism that can cause aspiration pneumonia [16, 17]. In this study, we chose four frequently encountered species: the bacteria *Staphylococcus aureus* FDA209P, *Streptococcus pneumonia* ATCC6305, *Pseudomonas aeruginosa* JCM2776, and the yeast *Candida albicans* NUD202. All organisms were maintained on brain-heart infusion agar (BHI; BD Biosciences, Rockville, MD, USA). The subcultures were freshly prepared before use. Each strain was cultured for 24 to 48 h in nutrient broth, and inoculated in the same broth at 37°C under aerobic or anaerobic conditions. Bacterial and fungal cells were harvested in the late logarithmic phase by centrifugation at 5,000 g at 4°C for 10 min and washed twice in phosphate-buffered saline (pH 7.2).

Bactericidal effects

Each bacterial strain or *C. albicans* (1×10^7 colony-forming units (CFU) /mL) was mixed with 1 mL of PBS, FW, ConCool F (containing 0.05% Chlorhexidine [CHG], Weltec Corporation, Osaka, Japan), or LISTERINE (containing methyl salicylate, cineole, thymol, and l-menthol, Johnson &Johnson, New Brunswick, NJ, USA) for 30 s. We used two concentrations of ConCool F: undiluted, and the optimal concentration suggested by the manufacturer (0.0006% CHG). After treatment, the mixture was diluted and 50 ul was plated on a BHI agar plate. The plates were inverted and cultured for 24 h in a 37°C incubator, after which the bacterial colony numbers were counted.

Measurement of the activity of superoxide dismutase

S. aureus (1×10^7 colony-forming units (CFU) /mL) were treated with 10, 30, 50, or 70% FW for 30 s. After treatment, we crushed the bacteria with Zirconia beads (NIPPON Genetics, Tokyo, Japan) and a Beads Crusher μT-12 (TAITEC, Saitama, Japan), and the superoxide dismutase (SOD) activity was measured using Superoxide Dismutase Assay Kits (Cayman Chemical, Ann Arbor, MI, USA). The absorbance was measured on a microplate reader model 3550 (BioRad, Hercules, CA). We counted the colony-forming units (CFU) of *S. aureus* treated with each concentration of FW.

Human cell culture

HeLa and HSC-3 cell lines were obtained from the Health Science Research Resources Bank (Osaka, Japan). The HeLa cells were maintained in minimum essential medium supplemented with 10% fetal calf serum (FCS), 50 mg/ mL streptomycin, and 50 U/mL penicillin in a 5% CO_2 incubator. The HSC-3 cells were maintained in RPMI1640 medium supplemented with 10% FCS, 50 μg/ml streptomycin, and 50 U/ml penicillin in a 5% CO_2 incubator.

Cell stimulation with FW and measurement of cytotoxicity

Both cell types were plated in 96-well plates at a two density (1×10^4 and 1×10^5 cells/well) on the day before the experiment, and treated with FW, ConCool F (undiluted only), or LISTERINE for 30 s. After treatment, the cells were washed with the appropriate cell culture medium and cultured for 1 h. The culture supernatant was harvested, and cell viability was measured with XTT Cell Proliferation Assay Kits (Cayman Chemical, Ann Arbor, MI, USA). We measured cytotoxicity as the cell viability after treatment.

Statistical analysis

The collected data were imported to SPSS ver. 26.0 (SPSS, Chicago, IL, USA) for statistical analysis. All experimental data are presented as the mean ± SD, and $n = 5$. Statistical analysis was performed using One Way Analysis of Variance with Tukey's multiple comparisons test; $p < 0.05$ was considered to indicate statistical significance. To evaluate the cytotoxicity of FW, we performed statistical analysis on the cell viability data only.

Results

Bactericidal effects

After treatment with FW, almost no colony formation was in any of the species investigated (Fig. 1a to d). The same results were found following treatment with LISTERINE and undiluted ConCool F. Diluted ConCool F produced bactericidal effects only on *C. Albicans*. (Fig. 1d).

SOD activity

We investigated the effect of FW treatment on the level of SOD activity in *S. aureus*, a major causative agent of aspiration pneumonia. SOD activity significantly increased more than twice when the bacteria were treated with 50% FW ($P < 0.001$), peaked at the point of 50% FW stimulating, and then decreased (Fig. 2a). Stimulating with 10% FW also showed a significant increase in SOD activity, but only slightly ($P = 0.017$, fold-change : 1.27)

A significant decrease colony formation was observed after treatment with 30% FW, and an extreme decrease in the number of bacteria was observed after treatment with 50% FW (Fig. 2b)

Cell viability

The three mouth rinses produced significantly lower cell viability ($P < 0.001$ for both cell density) in HeLa cells than in the control PBS-treated cells. There was no significant difference in the viability of HeLa cells between the three mouth rinses when we treated low-density cells. When stimulated high-density cells were treated, FW produced higher cell viability than that of the other mouth rinses (both $P < 0.001$) (Fig. 3a,b).

In HSC3 cells, the three mouth rinses produced significantly lower cell viability than PBS ($P < 0.001$ for both cell density). There was a significant difference between FW treatment and LISTERINE treatment in low-density cells. With treatment of high-density cells, the viability of FW was significantly higher than that of the other gargling agents (both $P < 0.001$) (Fig. 3c.d).

Discussion

There have been many studies on the use of FW in the field of dentistry. FW has been reported to be effective as an antimicrobial mouthwash in root canal treatment [18, 19]. There are, however, no reports of its use as a gargling agent for oral care of the elderly. Our results showed that FW may be valuable in the maintenance of oral hygiene. Aspiration pneumonia is caused by a wide range of bacteria. We analyzed the effect of FW on four major causative organisms of aspiration pneumonia, and found that it had excellent bactericidal effects against all four organisms. Its bactericidal effect was equal to or greater than that of conventional mouth rinses.

Several mechanisms of action have been reported for the bactericidal effect of FW. In the guidelines published by the Society for Oral Functional Water, it is suggested that its bactericidal mechanism may be based on oxidation by hypochlorous acid [20]. In this study, changes in the activity of SOD, which is an

indicator of oxidative reactions [21], was used to determine whether an oxidative response occurred when the bacteria were treated with FW. We confirmed a change in SOD activity in *S. aureus*, one of the primary causative bacteria of aspiration pneumonia. Since treatment of *Staphylococcus aureus* with 100% FW killed all bacteria, five different concentrations of FW were used for treatment, and SOD was measured when the bacteria survived treatment. Treatment with 50% FW significantly increased the SOD activity, and the concentration was consistent with the concentration at which a significant bactericidal effect on *Staphylococcus aureus* was observed. Following treatment with 70% FW, the SOD activity tended to be higher than that of the control, despite the small number of viable bacteria. These data supported the hypothesis that the bactericidal effect of FW arises from oxidative stress.

FW produced similar or lower rates of cell death as other mouth rinses. Especially, FW showed significantly higher cell viability than the other two mouth rinses in both cell lines with high-density cultures. This observation suggests that FW is less cytotoxic than the other gargling agents. Previous reports have shown that the use of FW has no adverse effects on the human body. Morita et al. reported that mice administered FW orally experienced no physical ill effects [22]. We envision that FW will be used for oral care of the elderly, and the fact that it is less cytotoxic than other gargling agents means that the elderly can use it safely.

The present experiment was preliminary, aiming to lay the groundwork for further clinical research, and is essentially a baseline report. However, the results suggest that FW may be more beneficial than other mouth rinses.

Chlorhexidine gluconate, which is the main component of ConCool F, is one of the most widely used mouth rinses globally [23, 24]. Most reports indicate that it is used in concentrations of 0.10–0.20% [25]. However, the concentration used varies from country to country. In Japan, the maximum concentration used is 0.05%, due to the risk of anaphylactic shock and gingival necrosis [26]. Further dilution is recommended; for ConCool F, the concentration after dilution is about 0.006%. CHG in Japan is diluted about 200 times more than the concentration used in other countries. It is not easy to directly quote the results of reports describing the effects of conventional chlorhexidine rinsing. Our results also show that, except for *C. albicans*, treatment with diluted CHG did not have an adequate bactericidal effect. FW is an aqueous solution consisting mainly of hypochlorous acid, which is purified by the electrolysis of salt water, and the possibility of gingival necrosis caused by FW is low. Although the stimulation time was short, Morita et al. also reported no gingival necrosis [22]. In our opinion, investigations into the safety of FW should be focused on aspects other than cytotoxicity. It has been reported that common mouth rinses, such as Listerine and ConCool F used in this experiment, have a pungent taste, which can cause taste disorders [27–29]. Taste disorders can cause a wide range of problems, and reduce an individual's quality of life. [30]. Many elderly individuals are hesitant to use gargle products under these circumstances. It is believed that taste disorders in gargling materials are mainly due to the effects of drugs [27]. FW is produced by the electrolysis of salt water, so the risk of taste disturbance is low. FW is also used for washing food, but no taste abnormalities have been reported due to washing. However, since it can be used for cleaning food, it appears that the risk of consuming FW is insignificant.

We are currently conducting a clinical study to determine the efficacy of FW use in the elderly. The subjects are instructed to gargle three times a day for one week, and the effectiveness of the treatment is examined. Although the study is still in progress and has only been conducted on 20 subjects, none have complained of taste abnormalities due to rinsing. A few subjects were bothered by the smell of hypochlorite chlorine, but none could not gargle (data not shown). In light of the above, FW may be a gargle material that can solve the problems faced by current gargle materials.

Although FW is a beneficial rinse agent, some clinical issues need to be addressed. First, the pH of FW is low (2.2–2.7), below the critical pH, and concern has been expressed about the demineralization of the teeth due to prolonged gargling. Tooth demineralization develops due to a lack of balance with tooth remineralization [31, 32]. We believe that the demineralization of teeth can be avoided by not allowing the oral pH to become acidic for long periods of time after rinsing with FW, by restoring it to neutral at an early stage. Therefore, after gargling with FW, it is essential to restore the pH of the oral cavity to neutral by rinsing with tap water. Morita et al. reported changes in crown morphology due to FW impregnation for three weeks in an in vitro experiment [22]. Still, no significant crown demineralization due to FW was observed in the short-term analysis. We may need to pay attention to gargling with FW in the long term.

FW has a short storage period, because it reverts to normal water after a period of time after purification [33], and is thus generally regarded as safe. However, this reversion indicates that the sterilizing power will be lost. FW maintains its function for one month in cold, dark storage after purification following the guidelines of the Japan Society for Oral Functional Water [20]. Ideally, FW should be used immediately after purification, so it is not easy to distribute effective FW in the market. Therefore, it is necessary to purchase a generating device for use. We aim to use FW in the oral care of the elderly, but since it is difficult to purchase a single device for each household, it is realistic to use it only in nursing homes and dental clinics. For FW use to become widespread in general households, it is necessary to downsize the generating device, but we believe that this problem can be solved in the future.

A variety of mouth rinses are available in the market, and are used for oral care. Our results confirmed that FW had a sufficient bactericidal effect and higher safety than other gargling agents. Safety is an essential factor in the use of gargles by the elderly. We believe that FW can become a new standard for gargling agents in the oral care of the elderly.

Conclusions

FW, produced by electrolysis of saline solution, had an excellent bactericidal effect against the causative organisms of aspiration pneumonia. The bactericidal effect was suggested to be related to its oxidative action. FW was found to be less irritating than conventional mouth rinse. These results indicate that the electrolytic acidic FW can be used as a new mouth rinse in the oral care of the elderly.

Abbreviations

FW
acid-electrolyzed Functional Water
ACC
Actual Chloride Concentration
CFU
Colony-Forming Units
CHG
ChlorHexidine Gluconate
SOD
Super Oxide Dismutase
FCS
Fetal Calf Serum.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors report no conflicts of interest related to this work.

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Authors' contributions

DO, KN, MT, MF did the practical work. DO wrote the manuscript, and KN, MA, TI discussed the practical steps and revised the manuscript. KN revised the statistics. All authors have read and approved the final manuscript.

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Figures

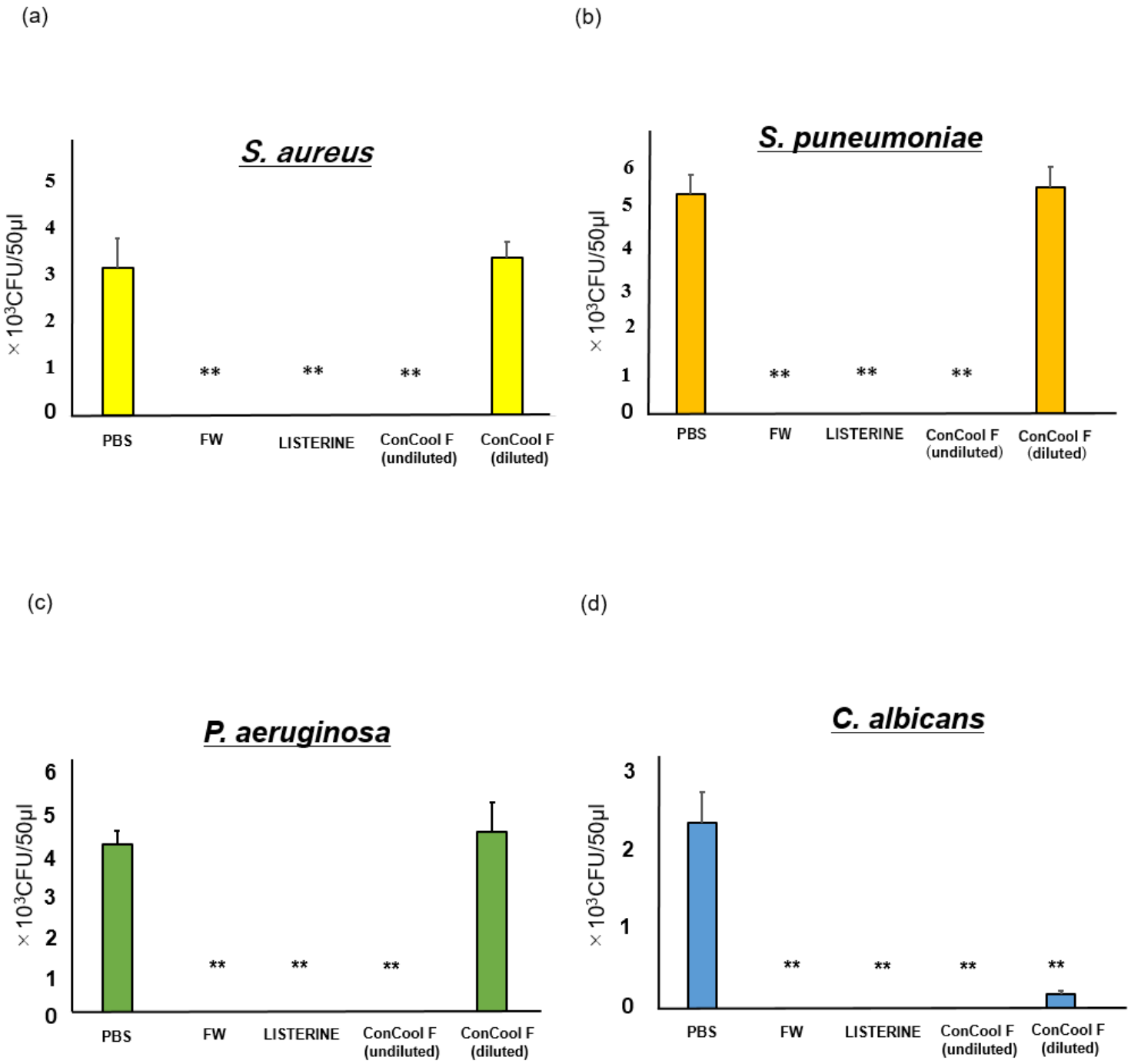

Figure 1

Bactericidal effects of each solution. The colony number was counted 24 h after plating, and the number of colony-forming units (CFUs) was counted.

(a) *S. aureus*, (b) *S. pneumoniae*, (c) *P. aeruginosa*, and (d) *C. albicans*. The data are expressed as mean ± SD. * P < 0.001 vs. PBS.

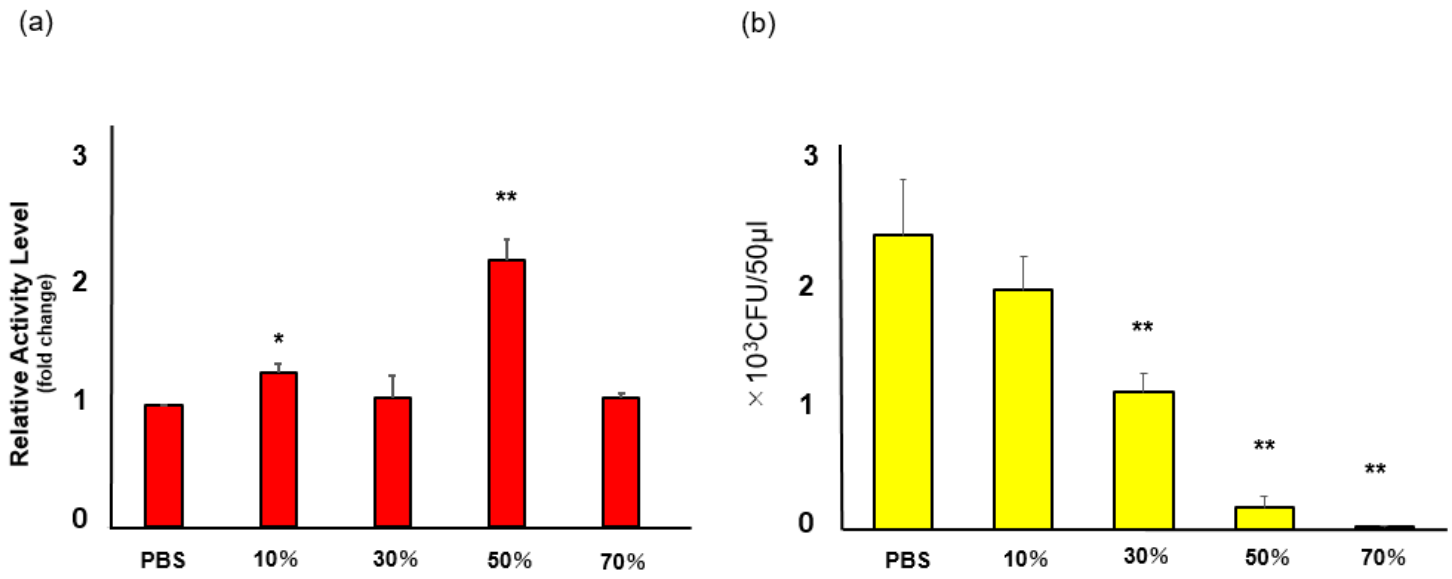

Figure 2

(a) Comparison of SOD activities of *S. aureus* after treatment with various concentrations of FW. (b) The colony numbers were counted 24 h after treatment, and the colony-forming units were calculated.

The data are expressed as mean ± SD from at least four independent experiments. * $P < 0.05$ vs. PBS. ** $P < 0.001$ vs. PBS.

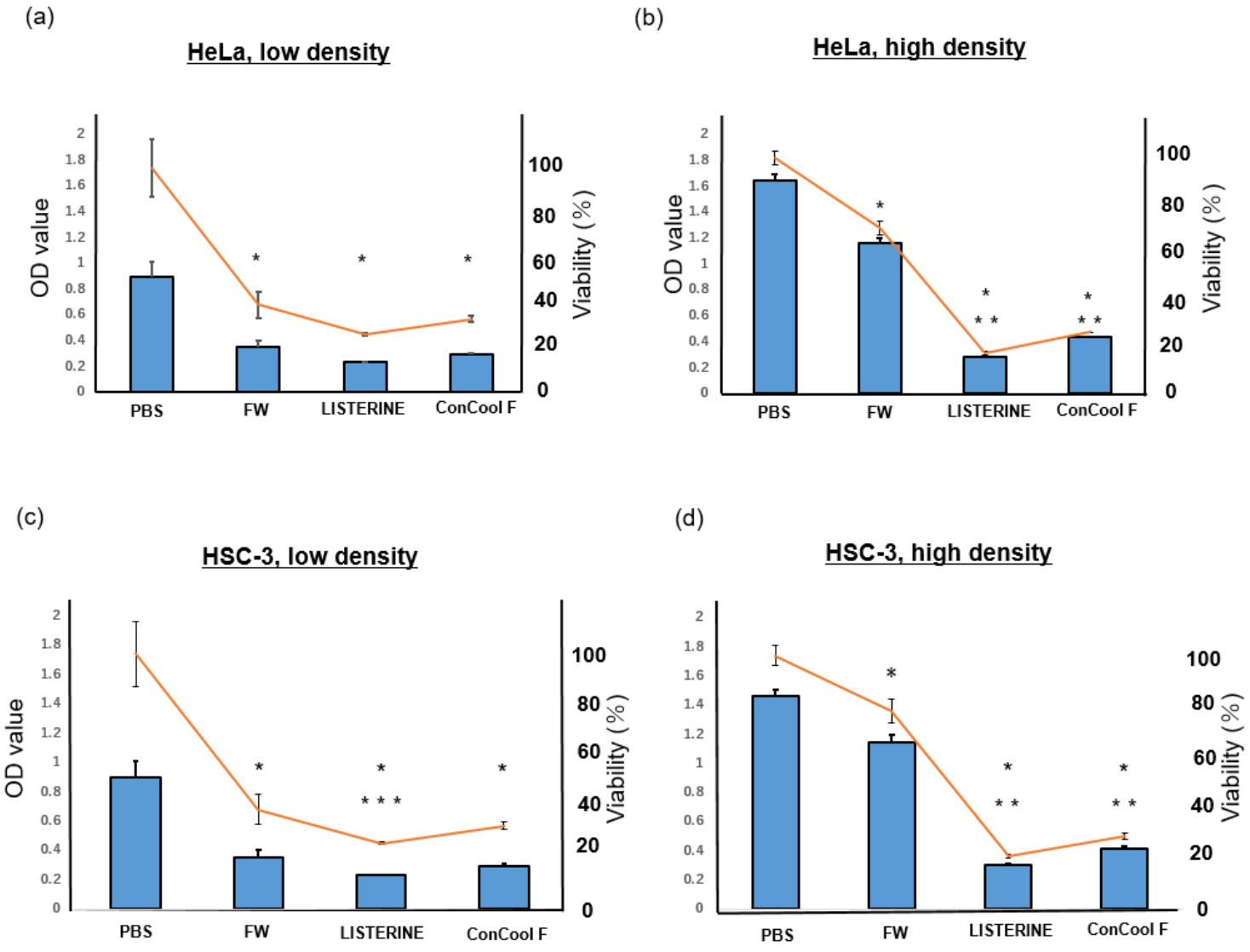

Figure 3

Viability of each cell after treatment. (a) HeLa, low density (1×10^4/well), (b) HeLa, high density (1×10^5/well). We show the OD score and cell viability; cell viability is shown as lines. (c) HSC-3, low density (1×10^4/well), (d) HSC-3, high density (1×10^5/well). We show the OD score and cell viability; cell viability is shown as lines. The data are expressed as mean ± SD. * P < 0.001 vs. PBS, * * P < 0.001 vs. FW, * * * P < 0.05 vs. FW.