Research Article

Microbubble-Mediated Ultrasound Enhances the Lethal Effect of Gentamicin on Planktonic Escherichia coli

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Received 22 January 2014; Revised 23 April 2014; Accepted 27 April 2014; Published 15 May 2014

Academic Editor: Konstantinos Anagnostakos

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Previous research has found that low-intensity ultrasound enhanced the lethal effect of gentamicin on planktonic E. coli. We aimed to further investigate whether microbubble-mediated low-intensity ultrasound could further enhance the antimicrobial efficacy of gentamicin. The planktonic E. coli (ATCC 25922) was distributed to four different interventions: control (G CON), microbubble only (G MB), ultrasound only (G US), and microbubble-mediated ultrasound (G MUS). Ultrasound was applied with 100 mW/cm² (average intensity) and 46.5 KHz, which presented no bactericidal activity. After 12 h, plate counting was used to estimate the number of bacteria, and bacterial micromorphology was observed with transmission electron microscope. The results showed that the viable counts of E. coli in G MUS were decreased by 1.01 to 1.42 log₁₀ CFU/mL compared with G US (𝑃< 0.01). The minimal inhibitory concentration (MIC) of gentamicin against E. coli was 1 µg/mL in the G MUS and G US groups, lower than that in the G CON and G MB groups (2 µg/mL). Transmission electron microscopy (TEM) images exhibited more destruction and higher thickness of bacterial cell membranes in the G MUS than those in other groups. The reason might be the increased permeability of cell membranes for gentamicin caused by acoustic cavitation.

1. Introduction

Antibiotics are commonly prescribed to cure diseases from epilolliculitis to fatal infections. However, bacteria are becoming increasingly resistant to antibiotics. In Europe, more than 25,000 patients die every year from infectious diseases because of multiresistant bacteria [1]. Antibiotic resistance is caused by many mechanisms, including reduced bacterial membrane permeability and gene mutations which change the targets of antibiotics and produce efflux proteins which pump the antibiotic out of the bacteria [2].

A series of literatures have found that low-intensity ultrasound enhances gentamicin killing of planktonic E. coli, while ultrasound alone does not kill bacteria [3–7]. It is suggested that stable cavitation of ultrasound might contribute to altering structure of bacterial cell membrane, thus facilitating the penetration of bacteria by antibiotics [8]. Our laboratory has found that low-intensity ultrasound enhances the antimicrobial efficacy of vancomycin against S. aureus in bone cements [9, 10]. Low-intensity ultrasound shows promise for enhancement of antibiotics actions for its easy access, noninvasiveness, and safety [2].

Recently microbubble-mediated ultrasound (MUS) has been increasingly used to improve the therapeutic effects of ultrasound in the fields of transdermal drug delivery, thrombolysis, and transfection of gene vectors [11, 12]. Microbubbles have a gas-filled structure, stabilized by a protein, lipid, or polymer shell; some microbubbles have been clinically approved as contrast agent. MBs can provide nuclei and lower the threshold for cavitation during sonication. Whether MUS enhances the bactericidal effect of gentamicin more than ultrasound (US) does is unknown. Therefore, we designed this study to investigate the in vitro response of planktonic E. coli to the combination of MUS and gentamicin.

2. Materials and Methods

2.1. Organisms and Antibiotic. Culture of Escherichia coli (ATCC 25922) was selected for this study, which was maintained on blood agar plates. 24 h before an experiment,
2.5 study. The diameter of MB is typically 1 to 8 μm. A MB contrast agent (Bracco, Milan, Italy) was used in this study. Figure 1 shows an illustration of the experimental apparatus for ultrasonic exposure. SonoVue, a microbubble, was reconstituted in distilled water without further purification. An inoculum was transferred from a colony on the agar plate to 10 mL of tryptic soy broth (TSB) without glucose (Oxoid, Basingstoke, UK) and grown overnight at 37°C. After 24 h, 0.01 mL of the bacteria fluid was transferred to 9.99 mL of sterile TSB on a rotary shaker and grown at 37°C. Growth curves showed that the bacteria were in the exponential phase of growth between one and ten hours. The number of bacteria in the suspensions was measured by serial dilutions in physiological saline solution (PSS) and plating onto nutrient agar. Plates were incubated for 24 h at 37°C. Gentamicin sulfate (Sigma, St. Louis, MO, USA) was reconstituted in 5 mL normal saline and the solution contained 2–5 × 10⁸ MBs/mL. The MB was reconstituted in 5 mL normal saline and the solution contained 2–5 × 10⁸ MBs/mL. The concentration of the initial solution was defined as 100% and the MB solution was further diluted in the TSB medium (1:9) in the MB only and MUS experiments; therefore, the final concentration was defined as 10% (v/v).

The ultrasonic generator (Nexus; Hexin Biomedical Devices, Hangzhou, China) was employed in these experiments with four ultrasonic transducers (operating at 46.5 KHz) in a bath. The bath was filled with water and maintained at 37°C. The temperature of the bacterial suspensions inside the tubes was monitored. Both the intensity (100 mW/cm²) as well as the frequency was calibrated by the manufacturer. The duty cycle was 1:3.

2.3. Measurement of MIC. The minimum inhibitory concentration (MIC) of gentamicin was determined as reported previously [3]. The MIC was measured by preparing a series of test tubes containing TSB and gentamicin at concentrations between 0 and 4 μg/mL (in 1 μg/mL increments). Each tube was inoculated with exponential growth-phase E. coli and incubated at 37°C for 24 h. Then, the concentrations of the cultures were measured by plate counting and the turbidities of the tubes were assessed. The MIC was defined as the lowest concentration of gentamicin that had viable counts of less than 10⁵ CFU/mL. The MIC for E. coli in this study was 2 μg/mL.

2.4. Bactericidal Activity. Frozen stocks were aerobically cultured onto blood agar plates at 37°C for 24 h. The bacterial suspension containing 10⁵ CFU/mL was prepared. 4 mL of bacteria suspension was placed into each tube. 0.5 mL of gentamicin or physiologic saline solution (PSS) was added to produce concentrations of gentamicin at 0, 1, or 2 μg/mL. And 0.5 mL of MB solution or PSS was added to produce concentrations of MB at 0%, 10%, or 20%. The planktonic E. coli was divided into control group (GCON), MB only group (GMB), US only group (GUS), and MB + US group (GMUS). And there were 3 subunits (n = 8) in each group according to the gentamicin concentrations of 0, 1, or 2 μg/mL. The tubes were placed in the bath. The ultrasonic transducers were placed 2 mm below each tube, and US was transmitted through the bottom of the tubes via a coupling gel. During experiments, the acoustic intensity (Average intensity) was set at 100 mW/cm². After 12 h of an experiment, samples were taken from each tube, serially diluted in PSS, and 100 μL was inoculated onto petri dishes containing nutrient agar by using the spread plate method. The petri dishes were incubated at 37°C for 24 h and counted. Parallel and identical sets of cultures were prepared at the time gentamicin and MB were introduced. The sets of US and MUS groups were placed in the sonicating bath, and the other sets were placed in an incubator. The mean and 95% confidence intervals of the log of the counts of colony forming units (CFU) per mL were calculated from the results of several replicate experiments.

2.5. Transmission Electron Microscope. After measurement of bactericidal activity, bacterial suspensions were transferred to examine ultrastructure of the bacteria. Histological assessment was conducted by electron microscopy. Bacteria suspensions were centrifuged at 5,000 rpm for 10 minutes. The liquid supernatant was removed and 4% glutaraldehyde was added on the pellet for fixation. The pellet was resuspended and stored at 4°C. Then, the bacteria were scanned by transmission electron microscope (JEM-1230, JOEL, Tokyo, Japan) to observe the microstructure. In order to quantify the alteration of bacterial wall, the thickness of bacterial cell wall was measured by using image analysis software (Image-Pro Plus 6.0, Media Cybernetics Inc., Silver Spring, MA, USA).

2.6. Statistical Analysis. The data of viable counts and thickness of cell wall were expressed as mean ± standard deviation. After Kolmogorov-Smirnov test for Gaussian distribution and a homogeneity test for variance were both passed, intergroup differences were compared by one-way analysis of variance (ANOVA) followed by the LSD post hoc test using SPSS version 16.0 (SPSS Inc., Chicago, IL, USA). A P value of < 0.05 was considered statistical significant.
3. Results

3.1. Acoustically Enhanced Bactericides. Figure 2 shows the mean viability (95% confidence intervals) of *E. coli* at 12 h of sonication. When no gentamicin (0 μg/mL) was added, there were no significant differences (F = 1.181, P = 0.335) between the GCON (8.45 ± 0.51) and the other three groups (GMb 8.81 ± 0.40, GUS 8.67 ± 0.24, and GSUM 8.69 ± 0.28). Without gentamicin, MB and low-intensity ultrasound were apparently insufficient to kill bacteria. At a gentamicin concentration of 1 μg/mL, the *E. coli* concentration in the GUS (6.86 ± 0.29) was significantly lower than that in the GCON (7.44 ± 0.64, P < 0.05); and GSUM was decreased to 5.44 ± 0.49, 1.42 log₁₀ CFU/mL (P < 0.01) less than the *E. coli* concentration of the GUS groups. Both GCON and GMb (7.19 ± 0.38) groups contained more than 10² CFU/mL and there was no significant difference (P = 0.304) between them. By contrast, both the GUS and GSUM groups had viable counts of less than 10⁷ CFU/mL, which indicated that the MIC decreased from 2 μg/mL to 1 μg/mL in the presence of ultrasound and/or microbubble.

At a gentamicin concentration of 2 μg/mL, the *E. coli* concentration in the GUS (3.89 ± 0.37) was lower than that in the GCON (4.45 ± 0.49, P < 0.05) and GMb (4.33 ± 0.46, P < 0.05) groups. Viable counts in the GSUM (2.88 ± 0.28) were further decreased by 1.01 log₁₀ CFU/mL (P < 0.01) compared with the GUS groups. The external microbubble (SonoVue) amplified the synergistic effect between ultrasound and antibiotics, which was observed in groups with gentamicin concentrations at 1 μg/mL and 2 μg/mL.

3.2. Microstructure of Bacteria. Figure 3 shows pictures of the ultrastructure of *E. coli*. At gentamicin concentration of 2 μg/mL, no pellet formation took place because of the low viable bacterial concentration. Therefore, only the bacteria exposed to gentamicin concentrations of 0 μg/mL and 1 μg/mL were examined by electron microscopy. In the GCON and GMb groups, the bacteria cell membranes were well preserved. In GUS and GSUM, partial destruction or disintegration of the cell membrane was detected. Figure 4 showed the mean thickness (nm) of bacteria cell walls in each group. When the gentamicin concentration was 0 μg/mL, the thickness of cell membranes in the GUS (24.38 ± 2.57) and GSUM (25.31 ± 3.83) groups was significantly higher than that in the GCON (17.00 ± 1.60, each P < 0.01) and GMb groups (17.24 ± 1.43, each P < 0.01). At a gentamicin concentration of 1 μg/mL, the thickness of cell membranes in the GUS (23.39 ± 3.83) and GSUM (26.10 ± 4.08) groups was significantly higher than that in the GCON (17.40 ± 1.74, each P < 0.01) and GMb groups (17.14 ± 1.93, each P < 0.01). There was no significant difference between GUS and GSUM groups in either of the two gentamicin concentrations: neither at 0 μg/mL (P = 0.658) nor at 1 μg/mL (P = 0.074).

4. Discussion

To our knowledge, the present study was the first to investigate the synergism of MUS and gentamicin regarding their lethal effect on planktonic *E. coli*. Our data indicated that MUS further enhanced the bactericidal effect of gentamicin on planktonic *E. coli* by more than one order of magnitude, compared with US while MUS alone did not reduce viability of bacteria. Furthermore, TEM images showed the partial destruction and greater thickness of the bacteria cell wall of GUS and GSUM, probably an explanation of the enhancing effect of MUS.

Advances in technologies have made US safely applicable for therapeutic purposes. Previous studies have shown synergism between US and antibiotics regarding their bactericidal effects (US alone is not bactericidal) [3, 4, 7, 8]. The mechanism of the synergism is complex. Runyan et al. argue that US could increase the permeability of bacterial cell membranes [14]. The term "sonoporation" is introduced to describe the temporal change in cell membrane permeability mediated by US [15, 16]. This change is successfully visualized in eukaryotic cell (HL-60) with electron microscope [17]. At a high acoustic pressure (> 150 mW/cm²), transient cavitation leads to microbubble implosion [14]. At a low acoustic pressure, however, microbubbles in medium oscillate in a symmetrical way, known as stable cavitation. Such stable oscillation creates liquid flow around the microbubbles, which is called microstream. When the oscillating microbubble is in close vicinity of the cell, the shear stress can cause pore formation on the cell membrane. In our study, the low intensity (average of 100 mW/cm²) mainly induced stable cavitation. The phenomenon of sonoporation may contribute to the penetration of the bacterial cell membrane by antibiotics.

However, the occurrence of sonoporation on bacteria cell walls needs to be substantiated by solid evidence, because
bacteria are much smaller and have a higher tensile strength than eukaryotic cell. Work by He et al. and Zhu et al. has indicated that MUS can enhance the antibiotic effect on biofilms of *S. aureus* and *S. epidermidis* [18, 19]. Since micropores are observed on the biofilms after MUS intervention, they believe that MUS might increase the biofilm permeability and stimulate the transport of antibiotics through the biofilm. Obviously, there is a difference between biofilms and planktonic bacteria. The bacteria in biofilms are sequestered in layers of exopolymers, which protect them from normal levels of antimicrobial therapy [20]. Ultrasonication significantly increases the transport of antibiotics through the biofilm [21], thus increasing their lethality.

However, the barrier between planktonic bacteria and antibiotics is the bacterial cell wall. Neither He et al. nor Zhu et al. provide evidence of sonoporation of bacteria cell walls [18, 19]. By contrast, our study might prove the occurrence of sonoporation in *E. coli* by using electron microscopy (Figure 3). Partial disruption and higher thickness of the bacterial cell walls in the G<sub>US</sub> and G<sub>MUS</sub> may be an indication of sonoporation.

The physical stress caused by cavitation decreased the stability of the bacterial outer membrane, then the bacterial membrane loosened after ultrasonic irradiation, and, consistent with previous study [22], the cellular walls appeared to be thicker than normal. Only in the G<sub>MUS</sub> group, discontinuity could be observed, a proof that MUS created a stronger cavitation than US alone. There was no significant difference regarding the mean thickness between the G<sub>MUS</sub> and G<sub>US</sub> groups, maybe because of the rapid decrease of the permeability [23]. As pores in the bacterial cell membranes reseal in the order of seconds after switching off the ultrasound [24], most of the small pores and disruption of cell walls had been resealed by the time the sample preparation for TEM was completed. However, we were able to observe some residual pores in the G<sub>MUS</sub> group with TEM in our study. Pore formation might be the main mechanism for small

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**Figure 3**: Structural images of bacteria. The structure of the bacteria of G<sub>CON</sub> and G<sub>MB</sub> groups was normal. The bacteria cell walls of G<sub>US</sub> and G<sub>MUS</sub> were wrinkled and thicker than those of G<sub>CON</sub> and G<sub>MB</sub>. Partial destruction (arrows) could be seen in the bacterial cell walls in the G<sub>MUS</sub> groups.
impermeable molecules [25]. We believed that sonoporation of the cell membranes of *E. coli* could have occurred and that this was amplified by external microbubbles (Figure 5). In our study, MUS further reduced viable bacteria and induced more destruction of the bacterial cell walls. We assumed that external MB, acted as cavitation nuclei, exerted additional microstream and shear stress on cell membranes, and created more pores during sonication. Microbubbles amplified the biophysical effect of US and thus possibly improved the drug transport into the bacteria. Ultrasound and MUS did not kill bacteria without antibiotics. The reason for this inability may be that the size of the pores created by low-intensity ultrasound is large enough to deliver the drug but not severe enough to create fatal damage [26].

US is an inexpensive, easy to handle, and noninvasive tool. The level of shear stress is largely dependent on US parameters [27], and we can adjust it with an acoustic pressure between 100 Pa and 1000 Pa [28]. According to Williams and Pitt [8], there appears to be an intensity threshold (between 100 and 10 mW/cm$^2$) below which ultrasound may not enhance the bactericidal effect of antibiotics. Pitt et al. establish that the viability of *E. coli* begins to decrease after 6 hours of sonication in combination with gentamicin [3]. An exposure to US irradiation with higher intensity and longer durations could accelerate the drug transport [5, 21]. However, it may cause skin damage [29]. As external microbubble could enhance the cavitation effect, lower intensity ultrasound with microbubble could be used to obtain equal efficacy and avoid tissue damage. Some studies have reported that low frequency US had a stronger bactericidal effect in the presence of antibiotics because the shear stress decreases as the frequency increases [30, 31]. In our model, we exposed *E. coli* solution to low frequency US at 100 mW/cm$^2$ for 12 hours and dramatic improvement was observed. The encouraging results might be due to the US parameters of low frequency, relatively high intensity, and long duration; all these US parameters were within the range of clinical physical therapy [32]. The exposure time of 12 hours is too long for therapeutic purpose. Further experiments are needed to reach an optimum combination of parameters.

We acknowledged certain limitations in the present study. (i) The TEM images are useful to investigate cell morphology after sonoporation. However, to achieve real-time and direct evidence, ultrafast imaging and fluorescently labeled marker molecules may be better techniques to study the interaction of cavitating microbubbles with bacteria in suspension. (ii) The concentration of microbubble was set at 10% (v/v). Therefore, further studies are required to reveal the threshold below which the ultrasonic effect is no longer significant. (iii) The results of the *in vitro* study of planktonic bacteria cannot be transferred to human beings. (iv) The antimicrobial efficacy
of the combination of MUS and antibiotic on bacteria and the involved cellular and molecular mechanisms deserve further investigation.

5. Conclusions

In conclusion, this research has demonstrated significant evidence that MUS, when combined with gentamicin, could further enhance the bactericidal effect and cause partial destruction of the bacterial cell wall. This technique may become a new treatment modality for bacterial infections.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

This work is supported by the National Natural Science Foundation of China (81101377, 81101345, 81171687, 81371954, 81201414, and 81201416), the Key Project of Zhejiang Provincial Department of Science and Technology (2011C13033), Zhejiang Provincial Natural Science Foundation of China (Y2100161, Y2090283), the Scientific Research Fund of Zhejiang Provincial Education Department (Y201018936), and The Fund of Health Department of Zhejiang Province (2012RCA032).

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