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Combination of therapeutic ultrasound with antibiotics interfere with the growth of bacterial culture that colonizes skin ulcers: An *in-vitro* study

Elaine Caldeira de Oliveira Guirro [a,*], Dejanira de Franceschi de Angelis [c], Natanael Teixeira Alves de Sousa [b], Rinaldo Roberto de Jesus Guirro [a]

[a] Department of Biomechanics, Medicine and Rehabilitation of the Locomotor System, Laboratory of Physiotherapeutic Resources, Ribeirão Preto Medical School of University of São Paulo, Ribeirão Preto, SP, Brazil
[b] Post-Graduation Program in Rehabilitation and Performance Functional, Ribeirão Preto Medical School of University of São Paulo, Ribeirão Preto, SP, Brazil
[c] Department of Biochemistry and Microbiology, Institute of Biosciences of Rio Claro of Paulista State University, Rio Claro, SP, Brazil

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ABSTRACT

Staphylococcus aureus and *Escherichia coli* are among the major bacterial species that colonize skin ulcers. Therapeutic ultrasound (TUS) produces biophysical effects that are relevant to wound healing; however, its application over a contaminated injury is not evidence-based. The objective of this research was to analyze the effect of TUS on *in vitro*-isolated *S. aureus* and *E. coli*, including the combination of ultrasound and antibiotics, in order to assess their antibiotic action on bacterial susceptibility. For the experiments, the bacterial strains were suspended in saline, then diluted (10^4 CFU/mL) for irradiation (at 1 and 3 MHz, 0.5 and 0.8 W/cm^2 for 0 and 15 min) and the combination treatment of ultrasonication and antibiotics was administered by adding nalidixic acid (*S. aureus*) and tetracycline (*E. coli*) at concentrations equivalent to 50% of the minimum inhibitory concentration (MIC). The experiments were carried out in duplicate with six repetitions. The suspensions were inoculated on to Petri plates and incubated at 37 °C and the colony forming units (CFUs) were counted after 24 h. The results were subjected to the Shapiro–Wilk normality test, followed by parametric ANOVA and Tukey's *post hoc* test at a significance level of 1%. The results demonstrated that the action of TUS at 1 MHz inhibited bacterial growth while at 3 MHz, bacterial growth was observed in both species. However, the synergistic combination of ultrasound and antibiotics was able to inhibit the growth of both bacteria completely after 15 min of ultrasonication. The results suggest that the action of ultrasound on *S. aureus* and *E. coli* are dependent on the oscillation frequency as well as the intensity and time of application. The combination of ultrasound with antibiotics was able to inhibit bacterial growth fully at all frequencies and doses in both species.

1. Introduction

The interaction of ultrasound with tissues can induce mechanical, chemical and thermal effects, depending on the equipment used as well as the established parameters, which in turn can lead to various biological effects [1]. It can interfere with the permeability of membranes by inducing the absorption of drugs, peptides and proteins. In general, these effects are related to transient permeabilization of the cell membrane mediated by ultrasound and are often attributed to biophysical effects, such as cavitation and acoustic microflow [2,3].

Therapeutic ultrasound (TUS) is a resource indicated for the treatment of wound healing [4]. However, the application of TUS is controversial when bacterial contamination is present.

Infections by microorganisms are one of the main complications in the healing process. *Staphylococcus aureus* (*S. aureus*) and *Escherichia coli* (*E. coli*) are among the major bacterial species that usually colonize skin ulcers [5], often developing into an infection or functioning as a reservoir of multidrug-resistant microorganisms [6]. However, *S. aureus* is the most common agent with a high level of virulence [7]; moreover, although some individuals do not develop clinical signs due to the presence of *E. coli*, this opportunistic microorganism can cause severe infections [8].

The behavior of bacteria after exposure to US is mainly evaluated at low frequencies and/or low intensities [9–11]; the frequency may range from 70 kHz to 10 MHz [12], but at low

* Corresponding author at: Faculdade de Medicina de Ribeirão Preto, 3900, Monte Alegre/SP, Ribeirão Preto 14049-900, Brazil.
E-mail address: ecguirro@fmrp.usp.br (E.C. de Oliveira Guirro).

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intensities (less than 10 mW/cm^2), US is sometimes associated with drugs [13]. However, the responses to radiation under the therapeutic ultrasound conditions (high frequency and high intensity) used in rehabilitation treatments are not yet established. The effects of therapeutic ultrasound on microorganisms and the increase in membrane permeability interfering with sensitivity to drugs are still not entirely explained. In view of the fact that *S. aureus* and *E. coli* are recognized as infectious agents of great importance, in this study, an attempt has been made to elucidate the deleterious biological effects induced by therapeutic ultrasound with different frequency and intensity, in the presence of absence of antibiotics.

2. Materials

In the experiment, ultrasound waves were generated at fundamental frequencies of 1 MHz and 3 MHz using a Sonacel ultrasound unit (Bioset Industry Electronic Technology®, Rio Claro/SP, Brazil). The ultrasound unit was calibrated by adjusting the acoustic pressure balance with an ultrasound power meter (model UPM-DT 10 – OHMIC Instruments, Easton, USA) before the start of each experiment.

Was evaluated nalidixic acid-resistant strains of *S. aureus* ATCC 6538 (Gram-positive) and *E. coli* BH100 lac +(Gram-negative). Liquid nutrient medium (Nutrient Agar; 5 mL) was inoculated with the stock cultures and incubated at 37 °C for 24 h with shaking at 150 rpm, then reinoculated every 15 days. The medium used for bacterial cultivation was brain heart infusion broth (BHI).

3. Methods

Initial tests performed to characterize the cultures with regard to antibiotic resistance using an antibiogram and the technique of agar diffusion [14] gave the antibiograms for each strain of microorganism regarding the following antibiotics: ampicillin, penicillin, oxacillin, tetracycline, kanamycin, lincomycin, erythromycin, and gentamicin. The results of the tests demonstrated that *S. aureus* and *E. coli* were more resistant to nalidixic acid and tetracycline, respectively.

Based on these findings, we performed an assay to determine the minimum inhibitory concentration (MIC) using the double serial dilution method. Nalidixic acid (Wintomylon – Sanofi Winthrop®) and tetracycline (Terramicina – Pfizer®) were prepared at various concentrations to determine the susceptibility of the bacteria towards these antibiotics [15].

The MIC was determined after 24 h of incubation of the inoculum at 37 °C by observation of the turbidity of the medium, which reflected the presence or absence of bacterial growth.

The MIC of nalidixic acid was found to be 0.48 mg/mL for *S. aureus*, whereas the MIC of tetracycline was 1.95 mg/mL for *E. coli*. Ultrasonic stimulation was applied to bacterial cultures without antibiotics as well as in association with them at approximately 50% of the MIC for each strain.

The bacterial strains were suspended in saline solution and then subjected to serial dilution until a concentration of 10^4 cells mL^{-1} was reached. To obtain the irradiated suspension of bacteria to, 330 mL of *S. aureus* and *E. coli* suspension was added in duplicate and plated on solid BHI medium before incubation for 24 h at 37 °C.

A system comprising a glass jar and magnetic agitator surrounded by a thermal bath was developed to expose the experimental samples to ultrasonic radiation. During irradiation, the system was immersed in a thermal bath with magnetic shaking to keep the suspension at a temperature of 33 ± 1 °C and to maintain homogeneity. An acrylic compartment with a lid was constructed, and holes made in strategic points to couple the transducer and thermometer, as shown in Fig. 1.

The suspensions of *S. aureus* and *E. coli* were irradiated with continuous ultrasound at intensities of 0.5 and 0.8 W/cm^2 (SATA), frequencies of 1 MHz and 3 MHz, for 0 (sham), 5 and 15 min, continuously (T0, T5 and T15, respectively). In the same way, the combination of ultrasound and antibiotics was administered by adding nalidixic acid (0.24 mg/mL) to the suspension of *S. aureus* and tetracycline (1.0 mg/mL) to the suspension of *E. coli*. The intensities used were selected on the basis of the results of pilot studies, which reported the absence of bacterial growth at intensities equal to zero or above 1.0 W/cm^2.

All experiments were performed in duplicate with six repetitions and after irradiation, the suspensions were plated on a total of 576 Petri dishes (9 × 15 mm) containing BHI medium, which were then inoculated with 100 μL of culture and incubated for 48 h at 36 °C. Next, the colonies were counted and expressed in

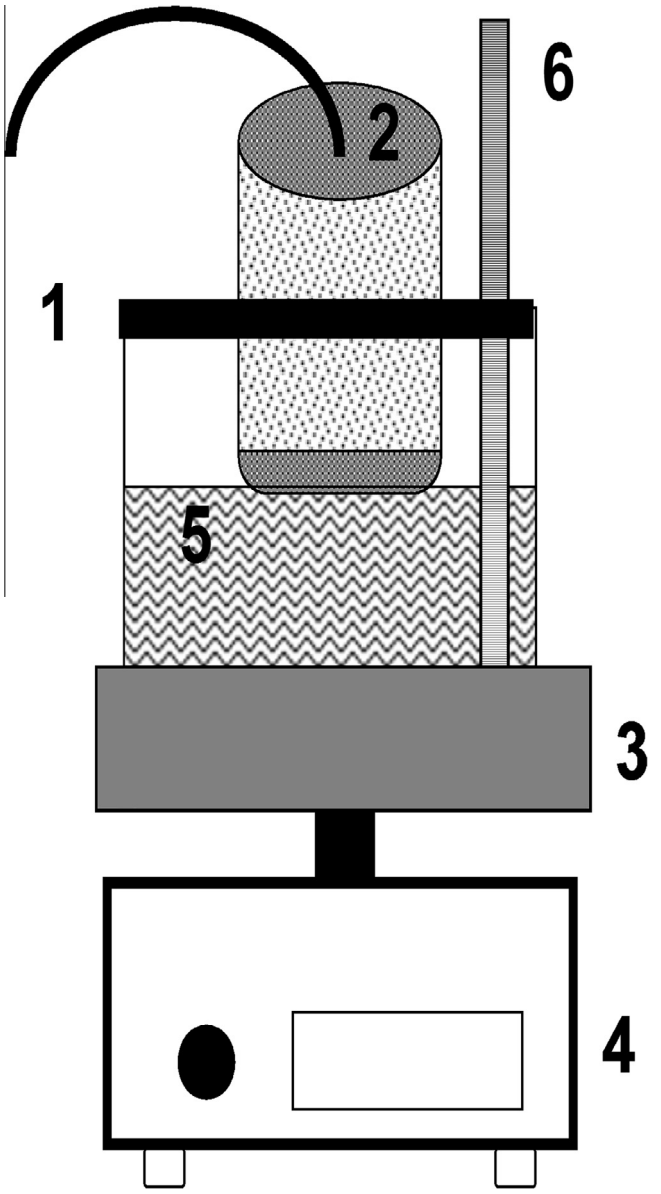

Fig. 1. Representative scheme of the system used for conditioning and irradiation of the samples. (1) Support for fixation of the transducer; (2) ultrasonic transducer; (3) thermal bath; (4) magnetic shaker, (5) samples in the solution; and (6) thermometer.

colony forming units (CFUs). The result was taken as the mean of two counts of the number of bacterial colonies in two consecutive dilutions and each measurement was taken 12 times.

The data collected were statistically analyzed using the software SPSS® 20.0 (IBM Corporation® – Chicago, IL, USA) in which Shapiro–Wilk's normality test, followed by parametric ANOVA and Tukey's *post hoc* test were applied at a significance level of 1%.

4. Results

With regard to the *S. aureus* strain, all of the experiments performed at a frequency of 1 MHz showed significant differences ($p < 0.001$) regarding the irradiation time, reducing bacterial growth and reaching total inhibition in the groups treated with a combination of ultrasound and antibiotics. On the other hand, bacterial growth was found to be stimulated at 3 MHz in the ultrasound groups treated without antibiotics. The treatment results obtained at the same irradiation time and frequency showed significant differences between the groups treated with ultrasound and the combination of ultrasound and antibiotics, although no significant differences were observed in terms of intensity regarding the latter. With regard to the frequency, significant differences were observed between all groups regarding the same irradiation time and intensity, except in the case of those treated with a combination of ultrasound and antibiotics (Table 1, Fig. 2).

Table 1
Mean (SD) of forming units of colonies *S. aureus* after exposure to ultrasound 1 or 3 MHz, at doses of 0.5 W/cm^2 or 0.8 W/cm^2, associated with nalidixic acid, at times 0 (sham), 5 and 15 min.

	0,5 W/cm^2			0,5 W/cm^2 + nalidixic acid			0,8 W/cm^2			0,8 W/cm^2 + nalidixic acid		
	T0	T5	T15	T0	T5	T15	T0	T5	T15	T0	T5	T15
1 MHz	21.25 (1.15)	17.73 (1.01) *	11.27 (0.89) *†	14.41 (0.53) #§	0 (0) *#	0 (0) *#§	20.69 (1.01)	18.23 (0.37)*	12.4 (1.81) *†#	14.2 (0.48) #§	0 (0) *#§	0 (0)*#§
3 MHz	22.65 (0.55)	24.1 (0.69) *‡	26.54 (1.19) *†‡	14.35 (0.43) #§	0 (0) *#	0 (0) *#§	22.53 (0.82)	24.64 (0.59)*‡	28.57 (1.51)*†#‡	14.08 (0.35) #§	0 (0) *#§	0 (0)*#§

$p < 0.01$ at the same dose and frequency in relation to time: * vs. T0; † vs. T5.
$p < 0.01$ at the same frequency and time in relation to dose: # vs. 0,5 W/cm^2; § vs. 0,8 W/cm^2.
$p < 0.01$ at the same dose and time in relation to frequency: ‡ vs. 1 MHz.

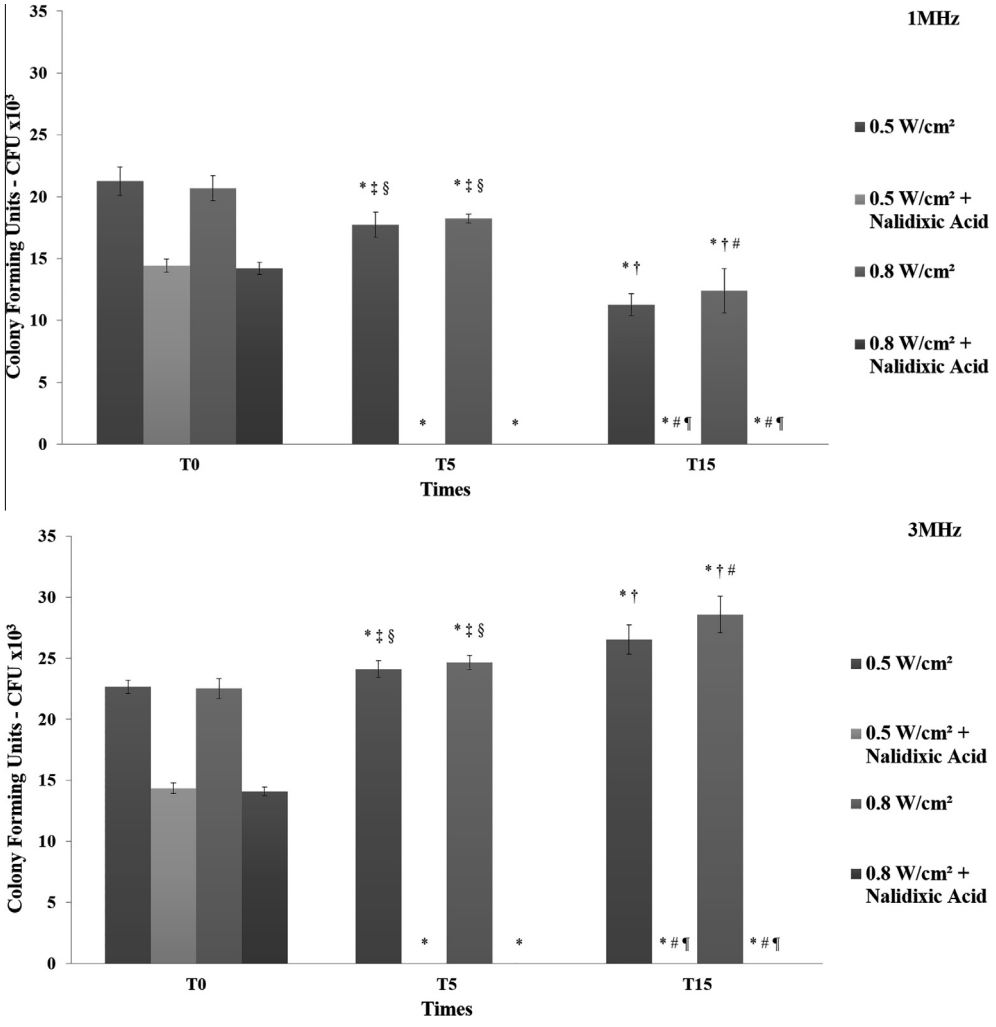

Fig. 2. Mean values (SD) growth of *S. aureus* before and after irradiated at frequencies of 1 and 3 MHz and intensities of 0.5 and 0.8 W/cm^2. Legends: T0 = pre-treatment (sham); T5 = 5-min treatment; T15 = 15-min treatment. * vs. T0 at the same dose. † vs. T5 at the same dose. ‡ vs. 0.5 W/cm^2 + nalidixic acid at T5. § vs. 0.8 W/cm^2 + nalidixic acid at T5. # vs. 0.5 W/cm^2 at T15. ¶ vs. 0.8 W/cm^2 at T5.

Table 2
Mean (SD) of forming units of colonies *E. coli* after exposure to ultrasound 1 or 3 MHz, at doses of 0.5 W/cm^2 or 0.8 W/cm^2, associated with tetracycline, at times 0 (sham), 5 and 15 min.

	0,5 W/cm^2			0,5 W/cm^2 + tetracycline			0,8 W/cm^2			0,8 W/cm^2 + tetracycline		
	T0	T5	T15	T0	T5	T15	T0	T5	T15	T0	T5	T15
1 MHz	23.64 (0.87)	20.85 (0.95) *	12.24 (0.7) *†	12.86 (0.7) #§	1.55 (0.16) *#§	0 (0) *†#§	23.15 (0.77)	18.88 (1.14) *	16.8 (1.26) *†#	12.9 (0.49) #§	1.5 (0.13) *#§	0 (0) *†#§
3 MHz	23.23 (0.74)	23.34 (0.72) ‡	24.56 (0.69) *‡	12.69 (0.35) #§	1.48 (0.16) *#§	0 (0) *†#§	23.23 (0.66)	23.5 (0.75) ‡	28.75 (0.88) *†#‡	12.95 (0.36) #§	0 (0) *#§¥‡	0 (0) *#§

$p < 0.01$ at the same dose and frequency in relation to time: * vs. T0; † vs. T5.
$p < 0.01$ at the same frequency and time in relation to dose: # vs. 0,5 W/cm^2; § vs. 0,8 W/cm^2; ¥ vs. 0,5 W/cm^2 + tetracycline.
$p < 0.01$ at the same dose and time in relation to frequency: ‡ vs. 1 MHz.

In the experiments performed with *E. coli* strains at a frequency of 1 MHz, the same result pattern as the one found for *S. aureus* was observed for treatment time. Nevertheless, bacterial growth was found to be increased in the groups treated with ultrasound, with total inhibition being observed in the groups treated with a combination of ultrasound and antibiotics at a frequency of 3 MHz after 15 min of treatment. The treatment results obtained at the same irradiation time and frequency were similar to those obtained for *S. aureus* in the comparison between groups treated with ultrasound and those treated with the combination of ultrasound and antibiotics. With regard to frequency, the results for *S. aureus* were found to be similar, except in the combination of ultrasound and antibiotics at doses from 0.5 to 0.8 W/cm^2 (Table 2, Fig. 3).

5. Discussion

Ultrasound has been used increasingly for medical purposes, which makes the investigation of its effects on biological systems important to elucidate the basic reaction mechanisms and establish safe levels for its application in this area.

In antibiotic therapy, the development of microbial resistance has already been found after treatment with a certain drug as microorganisms causing the disease develop specialized mechanisms that enable them to reproduce in a previously adverse environment. This phenomenon is considered the world's greatest problem and a substantial challenge in the management of healthcare resources [16]. Therefore, it is not possible to employ a single agent to eliminate or reduce microbiota.

In the present study, we observed that the use of ultrasound at a frequency of 3 MHz had the same effect on the growth of Gram-positive (*S. aureus*) and Gram-negative (*E. coli*) bacteria. However, different frequencies resulted in antagonistic responses: on the one hand, bacterial inhibition occurred in both strains at 1 MHz while on the other hand, bacterial growth occurred at 3 MHz. The effects of bacterial inhibition or multiplication in the face of ultrasonic irradiation were also observed by Iudin et al. [17], who correlated the findings with non-thermal effects.

The heating effect may also help by changing the physical state of membranes, making them more susceptible to deformation [2]. The results observed in our experiment demonstrated interference in bacterial growth mediated by ultrasound without the occurrence of a thermal effect, as the temperature was kept constant during irradiations.

There is a correlation between the acoustic cavitation and acoustic and bio microstreaming cellular effects [18]. The shear stress related to micro stream is relatively high compared with the stress associated with blood flow and when present at high levels, it can induce a broad spectrum of biological effects [19,20].

Machet and Boucaud [21] suggest that active ultrasound potentiates or increases the effectiveness of some antibiotics on the skin. The application of ultrasound combined with antibiotics *in vitro* should consider that the bacteria are in suspension and should start to move together with the culture medium and the antibiotic.

US can produce changes in cell membrane permeability, facilitating penetration of the antibiotic [22]. These mechanisms are still not fully understood; however, the possible influences of the various physical parameters should be considered, as observed in our study.

The mechanical and chemical effects arising from TUS may produce structural changes in proteins by enzymatic modification of sites necessary for proper operation of a lytic system, which can interfere with the permeability of membranes. Microstreaming induced by shear forces, chemical attack through the formation of radicals (H^+ and OH^-) and sonochemical degradation of hydrogen peroxide in water can interfere with bacterial viability [23,24].

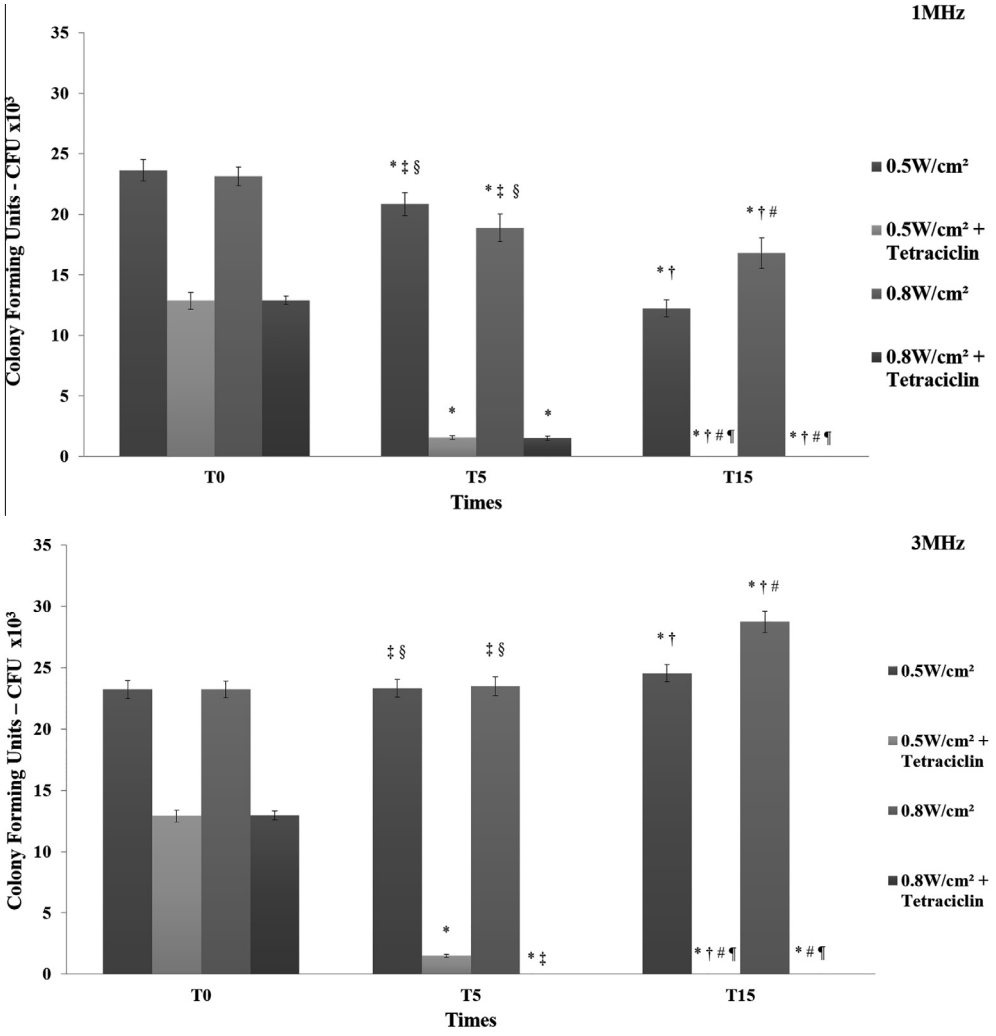

Fig. 3. Mean values (SD) growth of *E. coli* before and after irradiated at frequencies of 1 and 3 MHz and intensities of 0.5 and 0.8 W/cm^2. Legends: T0 = pre-treatment (sham); T5 = 5-min treatment; T15 = 15-min treatment. * vs. T0 at the same dose. † vs. T5 at the same dose. ‡ vs. 0.5 W/cm^2 + tetracycline at T5. § vs. 0.8 W/cm^2 + tetracycline at T5. # vs. 0.5 W/cm^2 at T15. ¶ vs. 0.8 W/cm^2 at T15.

Studies show that when US is applied in association with non-inhibitory concentrations of antibiotics, dose-related reductions in bacterial viability are produced [25,26]. In our study, complete inhibition of *S. aureus* growth was achieved using continuous ultrasound at frequencies of 1 and 3 MHz and at different doses in association with nalidixic acid.

However, complete inhibition was not achieved in *E. coli* strains after 5 min of irradiation (1 MHz + tetracycline), regardless of the dose used. These values did not differ after 15 min of application. By analyzing the effect of the combination of ultrasound and antibiotics on Gram-positive cultures (*S. aureus*), one can observe total inhibition at all doses and times tested, differently from Gram-negative bacteria (*E. coli*).

One study [27] assessed the effect of pulsed ultrasound, in association with human β-defensin 3 (hBD-3), on cultures of isolated *S. aureus* biofilms *in vitro*. The results demonstrated that the association of ultrasound with 50% MIC (hBD-3) was enough to produce inhibition. In the present study, we have achieved total inhibition using continuous ultrasound at different frequencies and doses in association with 50% MIC (nalidixic acid).

Another important point relates to the model proposed by Krasovitski et al. [28], where the model predicts the cellular membrane that is inherently capable of absorbing mechanical energy from the ultrasound field and transforming it into expansions and contractions of the intramembrane space. It further predicts that the maximum area strain is proportional to the acoustic pressure amplitude and inversely proportional to the square root of the frequency. The possibility of applying this model to explain the results presented in this study is justified by the similarity between animal and bacterial cell membranes. We hypothesize that the intramembrane space, between double lipid leaflets, increases and decreases in volume when exposed to US. The two layers are separated when the negative sound pressure exceeds the molecular attractive forces between the two leaflets and approaches positive pressure. For the authors, the double layer of the cell membrane would be able to turn the tide of oscillating sound pressure in intracellular deformation. The cyclical expansion and contraction of the lipid bilayer could activate mechanically sensitive proteins and/or increase the permeability of the membrane. This hypothesis is consistent with our findings and could explain the increase in activity when antibiotics were combined with US.

Although the effects produced by ultrasound on bacteria are in agreement with other findings, this is a subject that deserves further discussion. As to the diversity of resistant microorganisms, it is important to have better knowledge of the effects promoted by substances, which, in combination with physical agents, promote efficient antimicrobial action.

The results of this study found that the inhibition or growth of bacteria mediated by ultrasound is related to the physical parameters employed and that these, in association with the characteristics of the bacterial cell wall, may change bacterial sensitivity to antibiotics.

6. Conclusion

Different ultrasound parameters can produce different bacterial growth responses, producing either partial inhibition or increased CFUs. The combination of ultrasound and antibiotics was capable of inhibiting the growth of *S. aureus* and *E. coli* at all frequencies and doses, reaching total inhibition after 15 min of irradiation time, opening up new perspectives for the study of the combination of therapeutic resources in infected skin lesions.

Conflicts of interest

Not exist.

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