Ultrasonics Sonochemistry 32 (2016) 284–289

Contents lists available at ScienceDirect

Ultrasonics Sonochemistry

journal homepage: www.elsevier.com/locate/ultson

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

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ARTICLE INFO

Article history:
Received 14 April 2015
Received in revised form 22 March 2016
Accepted 23 March 2016
Available online 23 March 2016

Keywords:
Ultrasonic therapy
Physical therapy
Bacteria
Staphylococcus aureus
Escherichia coli

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

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http://dx.doi.org/10.1016/j.ultsonch.2016.03.024
1350-4177/

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

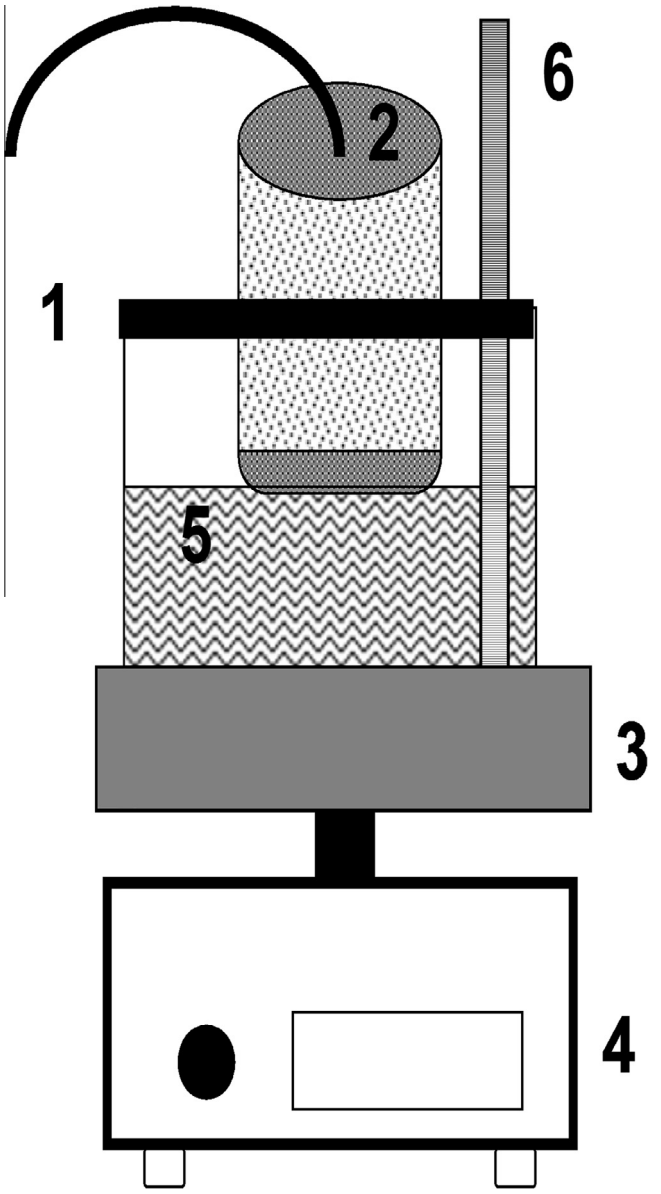

Fig. 1. Representative scheme of the system used for conditioning and irradiation of the samples. (1) Support for fixation of the transducer; (2) ultrasonic transducer; (3) thermal bath; (4) magnetic shaker, (5) samples in the solution; and (6) thermometer.