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SYNERGISM BETWEEN 1-MEGAHERTZ THERAPEUTIC ULTRASOUND PLUS CEFTAZIDIME ON GROWTH OF *PSEUDOMONAS AERUGINOSA*

NASIM KASHEF, [1]Ph.D., QORBAN BEHZADIAN NEJAD, [1]Ph.D., MANIJEH MOKHTARI DIZAJI, [*]Ph.D., AND MORTEZA SATTARI, [1]Ph.D.

From the [1]Department of Bacteriology and the [2]Department of Medical Physics, Tarbiat Modares University, Tehran, Iran.

ABSTRACT

Objective: The effect of ceftazidime on *Pseudomonas aeruginosa*, with or without application of 1MHz therapeutic ultrasound, was studied.

Method: An aqueous suspension of microorganisms in a sterile, sealed plate was placed in an ultrasonic tank operating at 1 MHz. Different power outputs were used. After desired time of exposure to the ultrasound, each sample was plated separately and after incubation, the number of colonies was counted.

Results: Results showed that ultrasound in combination with sMICs of ceftazidime was much more lethal to this bacterium than either of the treatments alone. The mechanism by which ultrasound enhances antibiotic action is due to the induction of uptake of antibiotic by perturbing or stressing the membrane.

Conclusion: This application of ultrasound may be useful for expanding the number of drugs available for treating localized infections by rendering bacteria susceptible to normally ineffective antibiotics.

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Keywords: Ceftazidime, *Pseudomonas aeruginosa*, Ultrasound

INTRODUCTION

Pseudomonas aeruginosa is an opportunistic human pathogen with innate resistance to many antibiotics and disinfectants, predominantly infecting patients with defects in antibacterial host defenses.[1]

The inactivation of microorganisms by ultrasonic waves was reported in the early 1930s.[2] A recent and relatively new application of ultrasound is in drug delivery. There are many reports in the literature suggesting that ultrasound activates, potentializes or makes more effective some pharmacological agents.[3-5] Jatzwauk et al. measured the germicidal efficacy of sonication, with or without chemical disinfectants, in an ultrasonic bath delivering a frequency of 35 kHz and an intensity of 0.66 W/cm^2 on different microorganisms. They found that sonication can act as a powerful synergistic agent to increase the cidal efficacy of the disinfectant against *S. aureus* and *P. aeruginosa*.[6] Qian et al. studied the effect of ultrasound on the antibiotic killing of bacteria in both planktonic and biofilm phenotypes. The enhanced antibiotic killing of *P. aeruginosa* and *E. coli* increased as

***Correspondence:** Nasim Kashef, Department of Bacteriology, School of Medical Sciences, Tarbiat Modares University, P.O.Box: 14115-111, Tehran, Iran. E-mail: kashefn@modares.ac.ir, Phone number: +98-21-88011001, Fax number: +98-21-88003030.

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the frequency of the insonation decreased.[7]

In this paper we report that therapeutic ultrasound enhances the effectiveness of ceftazidime against *P. aeruginosa* in vitro.

MATERIAL AND METHODS

Bacterial culture

The strain of *Pseudomonas aeruginosa* (ATCC 27853) was supplied by the Bu-Ali Sina Hospital Stock Collection. The bacteria were grown on Mueller-Hinton Agar at 37°C. After incubation for 24 h, a 10^5 CFU/mL suspension of microorganisms was prepared in physiological saline solution.

Antibiotic

The MIC of ceftazidime was determined by Macrodilution Test. Experiments were performed below the MIC at concentrations of 1 and 0.5 μg mL^{-1} ceftazidime.

Antibiotic treatment

50 μL of prepared suspension was diluted in 950 μL of PSS and 100 μL of it was plated on the following media: MHA contained 1/2 MIC, MHA contained 1/4 MIC, MHA contained MIC, and MHA .The two latter media were used as negative and positive controls, respectively. After incubation for 24 h at 37°C, the number of colonies was counted.

Ultrasound apparatus

In all experiments a therapeutic ultrasound (Model 434; Enraf Nanius Co., Netherlands) was used for sonication. A 1-MHz transducer was arranged in the walls of a 25 by 45 by 67-cm glass tank filled with deionized water. The temperature of the exposure tank water was controlled by a digital thermometer at 37±0.01°C.

Table I. Results of ceftazidime on *P. aeruginosa,* with or without application of 1-MHz therapeutic ultrasound.

Time (s)	Output (W)	Antibiotic (μg mL^{-1})	Mean±SD (CFU mL^{-1})
30	Sham	0	181.6±5.03
	0.25	0	138.6±9.60
		0.5	40.6±4.04
		1	37.3±3.51
60	Sham	0	201.3±10.59
	0.25	0	85.6±12.1
		0.5	22.3±3.51
		1	11.6±3.05
	0.5	0	0
	1	0	0
	1.5	0	0

Ultrasound treatment

Suspensions (10 mL) of *P. aeruginosa* in separate sterile, sealed polystyrene plates were placed in the deionized water-filled chamber and positioned on the beam axis nine centimeters from the ultrasound source. The samples were insonified for 30 and 60 s with an acoustic field produced by the 1-MHz transducer, operated at outputs of 0.25, 0.5, 1, and 1.5 W. The sample plates were rotated to ensure recirculation of the bubbles by acoustic radiation force. After exposure, each sample was plated separately and incubated for 24 h, after which the number of colonies was counted. There was a treatment control for each exposed sample. A sham-exposed sample was treated identically in series with the experimental sample, except that the acoustic field was turned off to provide a baseline for cell viability.

Combination of ultrasound and antibiotic treatments

This experiment was done as the previous one, but the suspensions of bacteria were prepared in solutions containing 1/2 and 1/4 MIC. Then, samples were insonified for 30 and 60 s and plated on MHA media containing 1/2 and 1/4 MIC, separately and incubated for 24 h at 37°C, after which the number of colonies was counted.

Statistical analysis

Comparisons between groups were done using either the Student's t-test or the ANOVA. Where significant differences were found for a particular variable, a multiple comparison test was carried out.

RESULTS

Determination of MIC value

The MIC level with this strain of *P. aeruginosa* is 2 μg mL^{-1}.

Effects of antibiotic treatment

Table I shows the results of ceftazidime on *P. aeruginosa*, with or without application of 1-MHz therapeutic ultrasound. Figure 1 shows the results of experiments performed at MIC and sMIC levels of ceftazidime. After treating the bacteria with antibiotic, 71 and 44% of bacteria were killed at 1/2 and 1/4 MIC concentrations, respectively.

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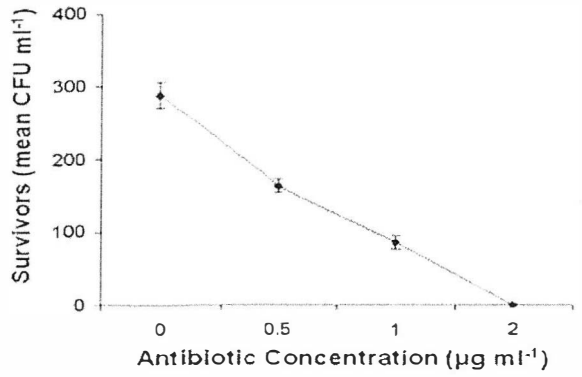

Fig. 1. Viability of 24 h culture of *P. aeruginosa* after exposure to ceftazidime. Error bars indicate 95% confidence interval.

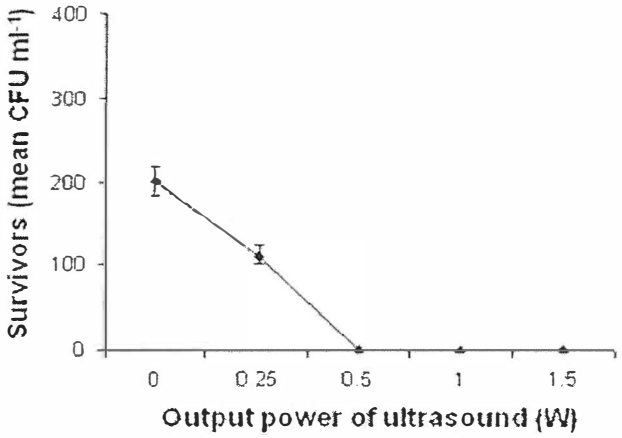

Fig. 2. Viability of 24 h culture of *P. aeruginosa* after exposure to ultrasound for 60s. Error bars indicate 95% confidence interval.

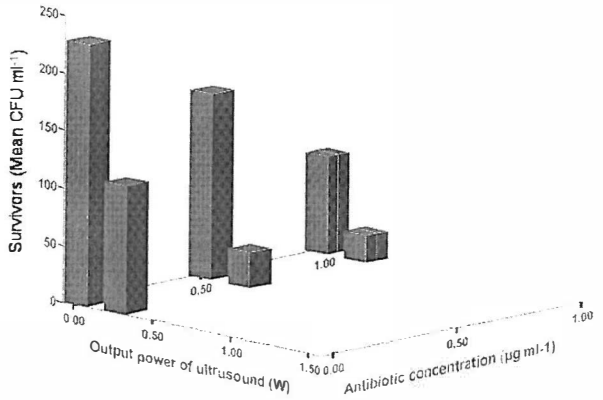

Fig. 3. Viability of 24 h culture of *P. aeruginosa* after exposure to combinations of ceftazidime and ultrasound.

Effects of ultrasound treatment

Figure 2 presents the results of experiments performed at different output levels of ultrasound. 100% of bacteria were killed by 60 s of sonication at 0.5, 1, and 1.5W, but sonication at 0.25W killed 71% of them. When the duration of exposure was reduced to 30 s, sonication at 0.25W killed 52% of bacteria.

Effects of combination of ultrasound and antibiotic treatments

Figure 3 presents the results of experiments performed at sMIC levels of antibiotic and output of 0.25 W. Combination of ultrasound and antibiotic treatments enhanced killing of bacteria compared to either treatments alone. A significant reduction of bacteria (96%) was found by 60 s of sonication at 0.25W and 1/2 MIC of ceftazidime.

DISCUSSION

With increasing antibiotic resistance among bacterial species, it is important to explore novel approaches to overcoming resistance mechanisms. The purpose of these experiments was to determine if therapeutic ultrasound in combination with antibiotic therapy would cause significant reduction in the number of viable bacteria.

Our results showed that the bactericidal effect of ceftazidime concentration equal to 1 µg mL^{-1} against this bacterium was more than that observed with 0.5 µg mL^{-1} and there was a 71% reduction in viable bacteria *in vitro* with 1/2 MIC of the antibiotic. Ultrasound treatment results showed that there was a significant effect of intensity for this bacterium, with percent killed increasing with increased intensity level ($p<0.000$) and a significant effect of time, with percent killed increasing with increased duration of exposure ($p<0.002$). These results are consistent with Schebra et al's. studies. They used a propagated ultrasonic energy at a frequency of 26 KHz to expose aqueous suspensions of bacteria (*E. coli, S. aureus*, *B. subtilis*, and *P. aeruginosa*) to evaluate the germicidal efficacy of ultrasound. They found that there was a significant effect of time and intensity for all four bacteria.[8]

The data in Fig. 3 show that the application of ultrasound enhances killing by the antibiotic. The most significant reduction of bacteria (96%) was found when bacteria were sonicated at 0.25 W and 1/2 MIC of ceftazidime for 60 s.

It has been found recently that the antimicrobial action of antibiotics may be substantially enhanced by simultaneous application of low frequency ultrasound, which acts synergistically with the antibiotic to kill bacteria both in suspensions and in biofilms formed on surfaces.[9] Rapoport et al. demonstrated that the efficiency of erythromycin in killing planktonic *P. aeruginosa* in-

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creased more than an order of magnitude upon the simultaneous application of ultrasound. Note that ultrasound alone does not kill the cells but rather sensitizes the cells to antibiotic action. They attributed this effect to the transient enhancement of membrane permeability to erythromycin.[3] Rediske et al. studied the effect of erythromycin on planktonic cultures of *P. aeruginosa*, with and without application of 70 KHz ultrasound. Ultrasound in combination with antibiotic reduced the viability of bacteria by 1-2 orders of magnitude compared with antibiotic alone. Electron spin resonance studies suggested that ultrasound induces uptake of antibiotic by perturbing or stressing the membrane.[10] Carmen et al. investigated the hypothesis that ultrasound increases antibiotic transport through biofilms of *Escherichia coli* and *Pseudomonas aeruginosa* using colony biofilms. They found that ultrasonication significantly increased transport of gentamicin across biofilms that normally blocked or slowed gentamicin transport when not exposed to ultrasound.[11]

Sonication is often used by investigators as a method of lysing bacterial cells. It depends upon bubble activity, heating, and the shear forces produced by the sonicator tip itself.[12,13] We also applied ultrasound at levels that had inhibitory effect on cultures of *P. aeruginosa*. So, according to other studies, the mechanism by which ultrasound killed the bacteria appears to be acoustic cavitation.

In our study, the mechanism by which ultrasound enhanced antibiotic action may be due to perturbation of the cell membrane or to stress responses by the bacteria.

This research has demonstrated significant evidence that therapeutic ultrasound, when combined with ceftazidime, reduces the number of viable bacteria in vitro. Obviously, there are many more refinements to be made to improve this procedure before it can be used clinically. But, this promising effect may result in developing a new methodology of killing resistant bacterial infections.

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