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Alteration of Bacterial Antibiotic Sensitivity After Short-Term Exposure to Diagnostic Ultrasound

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Abstract

Background: Many pathogenic bacteria show different levels of antibiotic resistance. Furthermore, a lot of hospital-acquired infections are caused by highly resistant or multidrug-resistant Gram-negative bacteria. According to WHO, patients with drug-resistant infections have higher morbidity and mortality. Moreover, patients infected with bacteria that are resistant to antibiotics considerably consume more healthcare resources.

Objectives: In this study, we explored a physical method of converting drug-resistant bacteria to drug-sensitive ones.

Materials and Methods: This is an in vitro case-control study, performed at the Ionizing and Non-ionizing Radiation Protection Research Center (INIRPRC), Shiraz University of Medical Sciences (SUMS), Shiraz, Iran in 2014. All experiments were carried out using Gram-negative bacteria *Klebsiella pneumonia* and *E. coli* and Gram-positive *Staphylococcus aureus* and *Streptococcus* group A, isolated from hospitalized patients. The bacterial strains were obtained from the Persian Type Culture Collection, IROST, Iran (*Klebsiella pneumonia* PTCC 1290) and Bacteriology Department of Shahid Faghihi Teaching Hospital, Shiraz, Iran (*E. coli*, *Staphylococcus aureus*, and *Streptococcus* group A). The bacteria in culture plates were exposed to diagnostic ultrasound using a MyLab70XVG sonography system for 5 minutes. Then, the bacteria were cultured on Mueller-Hinton agar and incubated at 35°C for 18 hours. Finally, antibiotic susceptibility test was performed and the inhibition zone in both control and exposed groups were measured. Three replicate agar plates were used for each test and the inhibition zones of the plates were recorded.

Results: Compared with the results obtained from unexposed bacteria, statistically significant variations of sensitivity to antibiotics were found in some strains after short-term exposure. In particular, we found major differences (making antibiotic-resistant bacteria susceptible or vice versa) in the diameters of inhibition zones in exposed and non-exposed samples of *Klebsiella pneumonia* and *Streptococcus*.

Conclusions: This study clearly shows that short-term exposure of microorganisms to diagnostic ultrasonic waves can significantly alter their sensitivity to antibiotics. We believe that this physical method of making the antibiotic-resistant population susceptible can open new horizons in antibiotic therapy of a broad range of diseases, including tuberculosis.

Keywords: Drug Resistance, Ultrasound, Infection, Antibiotics

1. Background

The World Health Organization (WHO) believes that antimicrobial resistance (AMR) is a progressive and serious threat to global public health that endangers the effective prevention and treatment of an ever-increasing range of infections caused by bacteria, parasites, viruses, and fungi. According to WHO, as AMR can be found in all parts of the world, international actions are needed to overcome this problem as new resistance mechanisms emerge and spread globally.

Diagnostic sonography as a very safe, reliable, and economic way to observe various organs of the body (1, 2) uses ultrasound waves in the frequency range of 1 - 20 MHz (however, frequencies up to 50 - 100 MHz have been used experimentally in ultrasound biomicroscopy, a technique used for obtaining high-resolution in vivo imaging of special regions of the body such as the anterior chamber of the eye). The annual number of ultrasound examinations has increased dramatically over the past decade.

The induction of "adaptive response" in bacteria has been already reported (3). Adaptive response can be defined as the induction of repair by pre-exposure to a low

level chemical or physical stress. We have previously shown that pre-exposure of living organisms to low levels of ionizing (4-7) or a large dose of non-ionizing radiation (8-12), decrease the detrimental biological effects on these organisms compared to exposure to the large dose alone. Therefore, adaptive response in bacteria can also be observed as the decrease in lethal effects of antibiotics after exposure to a low level physical stress such as short exposure to electromagnetic radiation or ultrasound.

2. Objectives

On the other hand, we have previously shown that pre-exposure of laboratory animals to non-ionizing electromagnetic radiation in radiofrequency (RF) range can induce a survival adaptive response which can be observed as increased resistance to a subsequent *Escherichia coli* infection (13, 14). Furthermore, over the past years, we have investigated the bio effects of physical stresses such as exposure to ultrasound for enhancing the sensitivity of bacteria to different antibiotics. This study aimed at developing an ultrasound-assisted method for increasing bacterial sensitivity to antibiotics.

3. Materials and Methods

3.1. Isolation and Identification of Isolates

This is an in vitro case-control study, performed at the Ionizing and Non-ionizing Radiation Protection Research Center (INIRPRC), Shiraz University of Medical Sciences (SUMS), Shiraz, Iran in 2014. The bacterial strains were obtained from the Persian Type Culture Collection, IROST, Iran (*Klebsiella pneumonia* PTCC 1290) and Bacteriology Department of Shahid Faghihi Teaching Hospital, Shiraz, Iran (*E. coli*, *Staphylococcus aureus* and *Streptococcus* group A).

The samples were cultured on blood agar and MacConkey agar for the isolation of microorganism. The culture plates were incubated at 37°C for 24 hours and observed for the presence or absence of visible bacterial growth.

3.2. Antibiotic Susceptibility Tests

We performed the antibiotic susceptibility tests by using the Kirby-Bauer disk diffusion method on Muller-Hinton agar (Figure 1). Drug susceptibility test was performed for nitrofurantoin, nalidixic acid (30 μg), gentamicin (10 μg), sulfamethoxazole, cephalexin, ciprofloxacin (5 μg), and cephalothin for Gram-negative bacteria and vancomycin (30 μg), erythromycin (15 μg), amoxicillin (20 μg), penicillin (10 Units), clindamycin (2 μg), and cefixime (5 μg) for Gram-positive bacteria. All culture media and antibiotic disks were purchased from Merck (Germany) and HiMedia Laboratories (Mumbai, India), respectively. Results for antibiotic susceptibility pattern before and after exposure to ultrasound were recorded and analyzed. The inhibition zone of each plate was recorded as the average of 2 diameters (mm) measured at right angles to one another. Three replicate agar plates were used for each regime. According to the CLSI guidelines (2013), the result were categorized as sensitive, intermediate, and resistance.

Figure 1. Antibiotic Susceptibility Test Performed by Using the Kirby-Bauer Disk Diffusion Method on Muller-Hinton Agar

3.3. Ultrasound Apparatus

The bacteria in culture plates were exposed to diagnostic ultrasound using a recently calibrated MyLab70XVG sonography system (EsaoteBiomedicaMyLab70XVG–Genova, Italia). All ultrasound exposures were performed by a 7.5 - 13 MHz linear array probe (type LA523) by an expert radiologist at Shahid Faghihi teaching Hospital, Shiraz, Iran.

3.4. Statistical Methods

The mean diameters of inhibition zones of the 3 replicates in exposed and non-exposed groups were compared using the nonparametric Mann-Whitney test. The significance level was considered at $P < 0.05$.

4. Results

Findings of this study are summarized in Tables 1 and 2. Compared to the results obtained from unexposed bacteria, statistically significant variations of sensitiv-

ity to antibiotics were found in some strains after short-term exposures. Tables 1 and 2 show the mean diameters of the inhibition zones of non-exposed *Klebsiella* and those exposed to diagnostic ultrasound in PenM, Res H and Doppler modes, respectively. This part of the study showed major differences in the diameters of zones of inhibition in exposed and non-exposed samples of *Klebsiella pneumonia* and *Staphylococcus aureus*. In two modes of ultrasound exposure (PenM and Doppler), ultrasonic waves made sensitive *Klebsiella pneumonia* resistant to cephalexin (P = 0.001). In ResH mode, ultrasound made sensitive *Klebsiella pneumonia* intermediate resistant to cephalexin (P = 0.011).

Tables 3 and 4 show the mean diameters of the inhibition zones of non-exposed *Staphylococcus epidermidis* and those exposed to diagnostic ultrasound in PenM, ResH and Doppler modes, respectively. Again, statistically significant variations of sensitivity to antibiotics were found in *Staphylococcus epidermidis* after short-term exposure to ultrasound. However, ultrasound was unable to make antibiotic-resistant bacteria susceptible or to make sensitive bacteria, resistant.

Tables 5 and 6 show the mean diameters of the inhibition zones of non-exposed *Staphylococcus aureus* and those exposed to diagnostic ultrasound in PenM, ResH and Doppler modes, respectively. As observed in previous tests, statistically significant variations of sensitivity to antibiotics were found in *Staphylococcus aureus* after short-term exposure to ultrasound. In this experiment, ultrasound was able to make antibiotic-resistant bacteria susceptible.

Table 1. The Mean Diameters of the Inhibition Zones (mm) of *Klebsiella* (PTCC: 1290) in Bacteria Exposed to Diagnostic Ultrasound (PEN M and RES H Modes) and Non-Exposed Bacteria[a]

Antibiotic	Bacteria Exposed to Diagnostic Ultrasound		Non-Exposed Bacteria		P Value
	Inhibition Zones[b]	Sensitivity	Inhibition Zones[b]	Sensitivity	
PenM Mode					
Nitrofurantoin	17.67 ± 0.58	Sensitive	16.34 ± 0.58	Intermediate	0.047
Nalidixic acid	20.34 ± 0.58	Sensitive	19.67 ± 0.58	Sensitive	0.230
Gentamicin	14.34 ± 0.58	Intermediate	14.67 ± 0.58	Intermediate	0.519
Sulfamethoxazol	20.67 ± 0.58	Sensitive	21.34 ± 0.58	Sensitive	0.230
Cephalexin	10.67 ± 1.15	Resistant	16.67 ± 0.58	Sensitive	0.001
Ciprofloxacin	19.67 ± 0.58	Intermediate	20.34 ± 0.58	Intermediate	0.230
Cephalothin	16.67 ± 0.58	Intermediate	18.34 ± 0.58	Sensitive	0.024
Res H Mode					
Nitrofurantoin	17.34 ± 0.58	Sensitive	16.34 ± 0.58	Intermediate	0.101
Nalidixic acid	19.34 ± 0.58	Sensitive	19.67 ± 0.58	Sensitive	0.519
Gentamicin	13.67 ± 0.58	Intermediate	14.67 ± 0.58	Intermediate	0.101
Sulfamethoxazol	20.34 ± 0.58	Sensitive	21.34 ± 0.58	Sensitive	0.101
Cephalexin	13.34 ± 1.15	Intermediate	16.67 ± 0.58	Sensitive	0.011
Ciprofloxacin	17.67 ± 0.58	Intermediate	20.34 ± 0.58	Intermediate	0.005
Cephalothin	16.34 ± 0.58	Intermediate	18.34 ± 0.58	Sensitive	0.013

[a](N = 3).
[b]Data are presented as mean ± SD.

Table 2. The Mean Diameters of the Inhibition Zones (mm) of *Klebsiella* (PTCC: 1290) in Bacteria Exposed to Diagnostic Ultrasound (Doppler) and Non-Exposed Bacteria[a]

Antibiotic	Bacteria Exposed to Diagnostic Ultrasound		Non-Exposed Bacteria		P Value
	Inhibition Zones[b]	Sensitivity	Inhibition Zones[b]	Sensitivity	
Nitrofurantoin	16.34 ± 0.58	Intermediate	16.34 ± 0.58	Intermediate	> 0.999
Nalidixic acid	18.34 ± 0.58	Intermediate	19.67 ± 0.58	Sensitive	0.047
Gentamicin	12.34 ± 0.58	Resistant	14.67 ± 0.58	Intermediate	0.008
Sulfamethoxazole	17.67 ± 0.58	Sensitive	21.34 ± 0.58	Sensitive	0.001
Cephalexin	12.34 ± 0.58	Resistant	16.67 ± 0.58	Sensitive	0.001
Ciprofloxacin	17.34 ± 0.58	Intermediate	20.34 ± 0.58	Intermediate	0.003
Cephalothin	16.34 ± 0.58	Intermediate	18.34 ± 0.58	Sensitive	0.013

[a](N = 3).
[b]Data are presented as mean ± SD.

In one mode of ultrasound exposure (PenM) ultrasound made resistant *Staphylococcus aureus* sensitive to amoxicillin (P = 0.003). However, ultrasound was unable to make antibiotic-resistant bacteria susceptible in other modes (ResH and Doppler). Tables 7 and 8 show the mean diameters of the inhibition zones of non-exposed *Salmonella* sp. and those exposed to diagnostic ultrasound in PenM, ResH and Doppler modes, respectively. Although statistically significant variations of sensitivity to antibiotics were found in *Salmonella* sp. after short-term exposure to ultrasound, conversion of antibiotic-resistant bacteria to susceptible or vice versa was not found.

Table 3. The Mean Diameters of the Inhibition Zones (mm) of *Staphylococcus epidermidis* in bacteria Exposed to Diagnostic Ultrasound (PenM and Res H modes) and Non-Exposed Bacteria

Antibiotic	Bacteria Exposed to Diagnostic Ultrasound		Non-Exposed Bacteria		P Value
	Inhibition Zones[a]	Sensitivity	Inhibition Zones[a]	Sensitivity	
Pen M Mode					
Vancomycin	16.34 ± 0.58	Sensitive	18.67 ± 0.58	Sensitive	0.008
Erythromycin	9.34 ± 0.58	Resistant	10.67 ± 0.58	Resistant	0.047
Amoxicillin	14.5 ± 0.5	Resistant	19.67 ± 0.58	Resistant	0.0001
Penicillin	17.34 ± 0.58	Resistant	19.67 ± 0.58	Resistant	0.008
Cefixime	9.84 ± 0.77	Resistant	9.34 ± 0.58	Resistant	0.417
ResH Mode					
Vancomycin	16.67 ± 0.58	Sensitive	18.67 ± 0.58	Sensitive	0.013
Erythromycin	10	Resistant	10.67 ± 0.58	Resistant	0.184
Amoxicillin	15.5 ± 0.87	Resistant	19.67 ± 0.58	Resistant	0.002
Penicillin	19.67 ± 0.58	Resistant	19.67 ± 0.58	Resistant	> 0.999
Cefixime	9.34 ± 0.58	Resistant	9.34 ± 0.58	Resistant	> 0.999

[a]Data are presented as mean ± SD.

Table 4. The Mean Diameters of the Inhibition Zones (mm) of *Staphylococcus epidermidis* in Bacteria Exposed to Diagnostic Ultrasound (Doppler) and Non-Exposed Bacteria[a]

Antibiotic	Bacteria Exposed to Diagnostic Ultrasound		Non-Exposed Bacteria		P Value
	Inhibition Zones[b]	Sensitivity	Inhibition Zones[b]	Sensitivity	
Vancomycin	16.34 ± 0.58	Sensitive	18.67 ± 0.58	Sensitive	0.008
Erythromycin	8.84 ± 0.29	Resistant	10.67 ± 0.58	Resistant	0.008
Amoxicillin	16.67 ± 0.58	Resistant	19.67 ± 0.58	Resistant	0.003
Penicillin	19.34 ± 0.58	Resistant	19.67 ± 0.58	Resistant	0.519
Cefixime	9.34 ± 0.58	Resistant	9.34 ± 0.58	Resistant	> 0.999

[a](N = 3).
[b]Data are presented as mean ± SD.

Table 5. The Mean Diameters of the Inhibition Zones (mm) of *Staphylococcus aureus* in Bacteria Exposed to Diagnostic Ultrasound (PenM and ResH Modes) and Non-Exposed Bacteria[a]

Antibiotic	Bacteria Exposed to Diagnostic Ultrasound		Non-Exposed Bacteria		P Value
	Inhibition Zones[b]	Sensitivity	Inhibition Zones[b]	Sensitivity	
Pen M Mode					
Vancomycin	17.67 ± 0.58	Sensitive	14.67 ± 0.58	Sensitive	0.003
Erythromycin	0	Resistant	8.34 ± 0.58	Resistant	0.002
Amoxicillin	20.34 ± 0.58	Sensitive	17.34 ± 0.58	Resistant	0.003
Penicillin	17.67 ± 0.58	Resistant	13.17 ± 0.77	Resistant	0.001
Clindamycin	29.34 ± 0.58	Sensitive	26.34 ± 0.58	Sensitive	0.003
Cefixime	15.17 ± 0.29(I)	ntermediate	16.84 ± 0.29	Intermediate	0.002
ResH Mode					
Vancomycin	16.67 ± 0.58	Sensitive	14.67 ± 0.58	Sensitive	0.013
Erythromycin	0	Resistant	8.34 ± 0.58	Resistant	0.002
Amoxicillin	17.5 ± 0.87	Resistant	17.34 ± 0.58	Resistant	0.795
Penicillin	16.67 ± 0.58	Resistant	13.17 ± 0.77	Resistant	0.003
Clindamycin	29.67 ± 0.58	Sensitive	26.34 ± 0.58	Sensitive	0.002
Cefixime	15.34 ± 0.58	Intermediate	16.84 ± 0.29	Intermediate	0.016

[a](N = 3).
[b]Data are presented as mean ± SD.

Table 6. The Mean Diameters of the Inhibition Zones (mm) of *Staphylococcus aureus* in Bacteria Exposed to Diagnostic Ultrasound (Doppler) and Non-Exposed Bacteria[a]

Antibiotic	Bacteria Exposed to Diagnostic Ultrasound		Non-Exposed Bacteria		P Value
	Inhibition Zones[b]	Sensitivity	Inhibition Zones[b]	Sensitivity	
Vancomycin	18.67 ± 0.58	Sensitive	14.67 ± 0.58	Sensitive	0.001
Erythromycin	0	Resistant	8.34 ± 0.58	Resistant	0.002
Amoxicillin	18.84 ± 0.29	Resistant	17.34 ± 0.58	Resistant	0.016
Penicillin	16.34 ± 0.58	Resistant	13.17 ± 0.77	Resistant	0.005
Clindamycin	29.5 ± 0.87	Sensitive	26.34 ± 0.58	Sensitive	0.006
Cefixime	15.5 ± 0.5	Intermediate	16.84 ± 0.29	Intermediate	0.016

[a](N = 3).
[b]Data are presented as mean ± SD.

Table 7. The Mean Diameters of the Inhibition Zones (mm) of *Salmonella* in Bacteria Exposed to Diagnostic Ultrasound (PEN M and RES H Modes) and Non-Exposed Bacteria[a]

Antibiotic	Bacteria Exposed to Diagnostic Ultrasound		Non-Exposed Bacteria		P Value
	Inhibition Zones[b]	Sensitivity	Inhibition Zones[b]	Sensitivity	
			PenM Mode		
Ciprofloxacin	28.34 ± 0.58	Sensitive	34.34 ± 0.58	Sensitive	0.0001
Cefixime	23.67 ± 0.58	Sensitive	25.67 ± 0.58	Sensitive	0.013
Amikacin	21.67 ± 0.58	Sensitive	21.67 ± 0.53	Sensitive	> 0.999
Sulfamethoxazole/ trimethoprim	26.67 ± 0.58	Sensitive	29.67 ± 0.58	Sensitive	0.003
Cephalexin	26.34 ± 0.58	Sensitive	24.34 ± 0.58	Sensitive	0.013
Gentamycin	20.67 ± 0.58	Sensitive	20.67 ± 0.58	Sensitive	> 0.999
			ResH Mode		
Ciprofloxacin	27.34 ± 0.58	Sensitive	34.34 ± 0.58	Sensitive	0.0001
Cefixime	22.34 ± 0.58	Sensitive	25.67 ± 0.58	Sensitive	0.002
Amikacin	20.67 ± 0.58	Sensitive	21.67 ± 0.53	Sensitive	0.101
Sulfamethoxazole/ trimethoprim	26.34 ± 0.58	Sensitive	29.67 ± 0.58	Sensitive	0.002
Cephalexin	23.17 ± 0.29	Sensitive	24.34 ± 0.58	Sensitive	0.035
Gentamycin	17.67 ± 0.58	Sensitive	20.67 ± 0.58	Sensitive	0.003

[a](N = 3).
[b]Data are presented as Mean ± SD.

Table 8. The Mean Diameters of the Inhibition Zones (mm) of *Salmonella* in Bacteria Exposed to Diagnostic Ultrasound (Doppler) and Non-Exposed Bacteria[a]

Antibiotic	Bacteria Exposed to Diagnostic Ultrasound		Non-Exposed Bacteria		P Value
	Inhibition Zones[b]	Sensitivity	Inhibition Zones[b]	Sensitivity	
Ciprofloxacin	27.67 ± 0.58	Sensitive	34.34 ± 0.58	Sensitive	0.0001
Cefixime	23.34 ± 0.58	Sensitive	25.67 ± 0.58	Sensitive	0.008
Amikacin	21.34 ± 0.58	Sensitive	21.67 ± 0.53	Sensitive	0.519
Sulfamethoxazole/ trimethoprim	28.84 ± 0.77	Sensitive	29.67 ± 0.58	Sensitive	0.206
Cephalexin	23.67 ± 0.58	Sensitive	24.34 ± 0.58	Sensitive	0.203
Gentamycin	18.5 ± 0.5	Sensitive	20.67 ± 0.58	Sensitive	0.008

[a](N = 3).
[b]Data are presented as mean ± SD.

5. Discussion

To the best of our knowledge this is the first study that explores the effect of ultrasound exposure as a mechanical stress on the antibiotic susceptibility of some microorganisms. In this study, we found some major alterations in the diameters of the inhibition zones in *Klebsiella pneumonia* and *Staphylococcus aureus* after exposure to ultrasound waves. Interestingly, ultrasound was capable of making some antibiotic-resistant bacteria susceptible as well as making some sensitive bacteria, resistant. Antibiotic resistance can be defined as the ability of microorganisms to resist the lethal effects of specific antibiotics. This phenomenon occurs when the effectiveness of drugs to cure or prevent infections reduces or vanishes (15). Lattimer et al. (16) in a paper published in JAMA in 1961 reported that in spite of great advances in medicine, scientists are losing the battle against drug resistance. At that time, they believed that the speed of discovery and development of new drugs was not fast enough to take over the significant ability of some microorganisms to develop resistant mutants. Therefore, they predicted that humans might encounter lethal epidemics in the future if they could not control drug-resistant microorganisms (16). Now, we should confess that the situation has not changed significantly since the publication of this paper more than 50 years ago.

The decrease observed in the diameters of the inhibition zones in *Klebsiella pneumonia* and *Staphylococcus aureus* after exposure to ultrasound waves, can be interpreted as an adaptive response. Adaptive response can be defined as the acquisition of radiation resistance against exposure to high dose in cultured cells or organisms which had been previously pretreated with an adapting low dose radiation (this low dose radiation is also called "priming dose" or "conditioning dose") (17). This observation is generally in line with our previous reports on the induction of adaptive response after exposure to low levels of ionizing (4-7) and non-ionizing radiation (8-12). More specifically, our findings are in line with the reports indicating that when bacteria are exposed to mild forms of different stresses (chemical and physical stresses), this stress improves their abilities to adapt and become resistant to any subsequent more extreme exposures (18-20). Also, that pre-exposure can increase the resistance to other exposures (e.g. exposure to antibiotics) and induce "cross-protection" phenomenon (3).

The main limitation of our experiment was the low number of bacterial strains studied. However, the unique interdepartment collaboration in our study was a significant strength point. Based on these results, we believe that short-term exposure of microorganisms to diagnostic ultrasonic waves can significantly alter their sensitivity to antibiotics. It can be concluded that the physical methods of making the antibiotic-resistant population susceptible can open new horizons in antibiotic therapy fora broad range of diseases, including tuberculosis. On the other hand, when exposure to ultrasound makes the antibiotic-susceptible population resistant, this may endanger patients' lives.

Footnotes

Authors' Contribution: Study concept and design: Seyed Mohammad Javad Mortazavi. Acquisition of data: Seyed Mohammad Javad Mortazavi, Leili Darvish, Mohammad Abounajmi, Sina Zarei, Tahereh Zare, Mohammad Taheri, and Samaneh Nematollahi. Analysis and interpretation of data: Seyed Mohammad Javad Mortazavi and Samaneh Nematollahi. Drafting of the manuscript: Seyed Mohammad Javad Mortazavi. Critical revision of the manuscript for important intellectual content: Seyed Mohammad Javad Mortazavi. Statistical analysis: Samaneh Nematollahi. Study supervision: Seyed Mohammad Javad Mortazavi.

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