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The effect of low-intensity pulsed sound waves delivered by the Exogen device on Staphylococcus aureus morphology and genetics

Exogen cihazı kaynaklı düşük yoğunluklu ses dalgalarının Staphylococcus aureus morfolojisi ve genetiği üzerine etkileri

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Amaç: Bu çalışmada ortopedik cerrahide kaynama gecikmesi ya da kaynamama durumlarında kullanılması önerilen Exogen cihazı ile verilen düşük yoğunluklu ses dalgalarının, ortopedik enfeksiyonlarda sık karşılaşılan *Staphylococcus aureus*'un koloni sayısı, antimikrobiyal duyarlılığı, bakteriyel morfolojisi ve genetiği üzerine etkisi araştırıldı.

Çalışma planı: İçinde *S. aureus* (ATCC 25923) suşu bulunan 0.5 McFarland bulanıklığında hazırlanan 30 adet tüp süspansiyonunun 15'ine (test grubu) 20 dakika boyunca Exogen cihazı ile düşük yoğunluklu ses dalgası uygulanırken, diğer 15'ine (kontrol grubu) ses dalgası uygulanmadı. Uygulama sonrasında iki gruptaki tüpler koloni sayısı, antibiyotik duyarlılığı ve genotip farklılıklar açısından karşılaştırıldı. Grupların histolojik değerlendirmeleri elektron mikroskopu ile yapıldı.

Sonuçlar: Uygulama sonrasında, kontrol grubuyla karşılaştırıldığında, test grubundaki bakteri koloni sayısı anlamlı derecede azalmış bulundu ($p<0.001$). İki grup arasında antibiyotik duyarlılığı ve genotip değişim açısından anlamlı fark bulunmadı. Kontrol grubundaki bakterilerin hücre duvar yapıları normal bulunurken, test grubunda hücre duvarlarında kısmi yıkım veya parçalanma görüldü. Bakteri hücre duvar kalınlığı test grubunda kontrol grubuna göre anlamlı derecede fazla idi (sırasıyla, 41.54 nm ve 24.27 nm, $p<0.001$).

Çıkarımlar: Düşük yoğunluklu ses dalgasının, ortopedik cerrahide enfeksiyonun önlenmesi için profilaktik olarak ya da enfekte kaynamamaların tedavisinde adjuvan bir yöntem olarak kullanılması düşünülebilir.

Anahtar sözcükler: Bakteri/analiz/genetik; koloni sayımı, mikrobiyal; Staphylococcus aureus; ultrasonik tedavi.

Objectives: We investigated the effect of low-intensity pulsed sound waves delivered by the Exogen device, which is recommended for the treatment of delayed union and nonunion in orthopedic surgery, on the colony number, antimicrobial susceptibility, bacterial morphology, and genetics of *Staphylococcus aureus,* which is a frequent pathogen in orthopedic infections.

Methods: Thirty tubes containing 0.5 McFarland suspensions of *S. aureus* (ATCC 25923) were used. Fifteen tubes forming the test group were subjected to low-intensity sound waves by the Exogen device for 20 minutes. The remaining 15 tubes were untreated as controls. The two groups were then compared with respect to colony number, antibiotic susceptibility, and genotypic properties. The tubes were examined histologically by electron microscopy.

Results: The test tubes treated with sound waves showed a significantly lower number of bacteria colonies compared to the control tubes ($p<0.001$). The two groups were similar with respect to antibiotic susceptibility and genotypic properties. Bacterial cell wall structure in the control group was of normal appearance, whereas partial destruction and break-up were observed in test samples. Bacterial cell wall thickness was significantly higher in the test group compared to the control group (41.54 nm and 24.27 nm, respectively; $p<0.001$).

Conclusion: Low-intensity sound waves may be beneficial as a prophylactic measure to prevent infections in primary orthopedic operations and as an adjuvant therapy for infected nonunions.

Key words: Bacteria/analysis/genetics; colony count, microbial; Staphylococcus aureus; ultrasonic therapy.

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Investigations about fatal effects of sound waves on microorganisms started during 1920s.[1] For this purpose, sound waves are used in food sector, sterilization of surgical instruments, disinfection of waste water, elimination of the biofilm membranes on medical instruments and enhancement of antibiotic susceptibility.[2-4] Ultrasonic waves produced throughout the ultrasonic process cause consequtive compressions and expansions, which might be responsible for the destruction of their cell membranes, localized heating, production of free radicals and acoustic cavitation on microorganisms In addition to these benificial effects of ultrasound, its genotoxic effect was shown on healthy tissues of human beings and animals.[5-9] But as far as we are acquinted with the literature its genotoxic effect on Staphylococcus aureus (S. aureus) is not mentioned.

In our study we investigated whether any change in genetic or morphologic structure or, antibiotic susceptibility occurs in colonies of S. aureus exposed to low intensity pulsed ultrasound (LIPU).

Material and methods

Thirty tubes containing S.aureus (ATCC 25923) stains which were prepared in microbiology laboratuary of our hospital were devidedinto two equal groups, test group (n=15)andcontrol group respectively(n=15). We applied LIPU by the Exogen® device on 15 tubes in test group during 20 minutes of exposure time, while remaining 15 tubes which were controls were except this procedure. Temperature changes during LIPU application were monitored with a K-type thermocouple.[10] After LIPU application to bacteria suspensions they were sent to laboratuary of histology for investigation of bacteria's structure. Colony counts, antibiotic susceptibility, genotipical and histological measurement results were compared between two goups at the end of the study.

Preparation of bacteria colonies

In this study S.aureus (ATCC 25923) was used as test microorganism. The lyophilized bacteria stock was inoculated on Trypticase Soy Broth (BBL,USA) and incubated at 37 ºC for 16-18 hours. After incubation, subcultures were made on blood agar plates and incubated at 37 ºC for 24 hours. A Gram stained was performed from a pure colony grown on blood agar plate to control the purity of the culture. Colonies of S. aureus standard stain were suspended in Trypticase Soy Broth at MacFarland Standard 0,5 turbidity using flat bottommed tubes. Broth culture was allocated into 6 tubes (3 for test group, 3 for control group) equally each including 0,5ml aliquots of the broth culture. Contamination from infectious aerosols was prevented keeping the tubes closed during the process.

Aplication of low intensity pulsed ultrasound by Exogen® Device

A commercially available therapeutic ultrasound device (Exogen 3000; Smith & Nephew Inc, Memphis, TN, USA) was used as an ultrasound source(Exogen 3000; Smith & Nephew Inc, Memphis, TN, USA). This ultrasound device was of low intensity(30-161 mW/cm2) and high frequency(1.5 mHz).

After applying coupling jel under each tube, the probe of LIPU source was set LIPU was applied 20 minutes for each tube while the tubes were under heat control The procedure was repeated for 5 times with different tubes until total number of tubes in and control groups amounted to 15.

Coupling gel was used to ensure effective transfer of the acoustic pressure wave to the bacteria in tubes. Temperature changes during ultrasound treatments were monitored with a K-type thermocouple.[10]

Microbiologic evaluation

After ultrasound treatment, bacterial solution from each tube(both test and control tubes) was inoculated on to blood agar plates using calibrated loops and incubated at 37 ºC for 24 hours. The Colony Forming Units per mililiter (CFU/ml) of bacterial suspension was determined using Cultural Colony Count Method on Agar Plate.

Antimicrobial Susceptibility of the strains to Penicilin [(10 U) Oxoid, UK], Oxacillin [1μg Oxoid,UK], Teicoplanin [30μg Oxoid,UK], Erythromycin [15μg Oxoid,UK], Clindamicine [2μg Oxoid,UK], Levofloxacine [5μg Oxoid,UK], Vancomycine [30μg Oxoid,UK], and Ciprofloxacin [5 μg Oxoid,UK] was investigated by a standardized disk-diffusion method performed on Mueller-Hinton agar, according to Kirby-Bauer suggestions and following the criteria of National Committee for Clinical Laboratory Standards (NCCLS).[11] While determination of antimicrobial susceptibility status of stains, zone diameters were not recorded.

Genotypic discriminations of all strains (in test group and control group) were performed by Arbitrarily Primed Polymerase Chain Reaction (AP-PCR). DNA isolation and extraction were performed according to protocol which proposed by Guducuoglu et al.[12] We used to universal primer M13 (5'-GAGGGTGGCGGTTCT-3') in amplification.[13] PCR products were visualized under UV light by a naked eye on a 1.8 % agarose gel with etidium bromide. Band profiles in strains were evaluated according to Dice coefficient.[14] Strains which coefficient of similarity up to 90% same genotype, between 70-90 % subtypes and lower from 70% different genotype were accepted.

Histological assessment

After 20 minutes of ultrasound treatment, bacterial suspensions were transferred to histology laboratory in order to examine ultrastructural morphology of the bacteria. Histological assessment of the microorganisms were conducted by electron microscopy.

Bacterium suspensions were centrifuged for 5 minutes at 5000 rpm. Following the removal of supernatant, 2 cc %2,5 gluteraldehyde was added on the pellet for fixation. Pellet was resuspended and left at 4°C for 4 hours. After last centrifugation the pellet was dropped onto prewarmed slides and approximately the same amount of preheated % 2 agar was added onto it and the mixture was stirred at 60°C. At room temperature they became solid and were cut into 1mm3 pieces with razor blade. Then they were embedded in epoxy resin and left at 70°C 12 hours for polymerisation. With Leica®, Ultramicrotome(Leica Microsystems GmbH, Vienna_ AUSTRIA) 70 nm slides were obtained and stained with uranyl acetate and lead nitrate. The photographs were taken by Megaview III®, (Olympus Soft Imaging Solutions GmbH, Münster-GERMANY) Digital camera attached to JEOL, JEM-1011 (Jeol Ltd. Tokyo-JAPAN) Electron Microscope. Bacterial wall thicknesses were measured from randomly selected from 13 test and 15 control groups bacteria by using a graphic editing software (iTEM 5.0© Soft Imaging Systems, GmbH, Münster, Germany).

Statistical analysis

Statistical analysis of the study was performed using SPSS 10.0 software(SPSS Inc. Chicago, Illinois-USA). Data were analysed using one- sample t test. Significance was set at $p<0.05$.

Results

In the test group, there was a significant reduction in viable bacteria count due to ultrasound treatment with approximately 5 log10 CFU/ml to 3.66 log10 CFU/ml ($P<0.001$).

No resistance to penicillin, oxacillin, teicoplanin, erythromycin, clindamicine, levofloxacine, vancomycin, or ciprofloxacin has been encountered in antimicrobial susceptibility tests. It was found that LIPU application did not cause any genetic differences detectable by AP-PCR process (Fig.1). No significant change occured statistically during follow-up of heat between groups (Control group: 24.4±0.5 °C, test group: 23.2±0.6 °C, $P=0.35$).

Electron microscopical histologic evaluation yielded the following results: In the control group, the cell wall ultrastructures of bacteria were preserved well and many of the bacteria were seen at mitosis. In the electron micrographs of test group with LIPU, partial destruction or disintegration of the cell walls were detected in some bacteria (Fig.2).

Mean wall thicness of bacteria in test group greater than control group was determined. And this finding there was a significant as statistically. (sequential, 41.54nm ve 24.27nm,$p<0.001$).

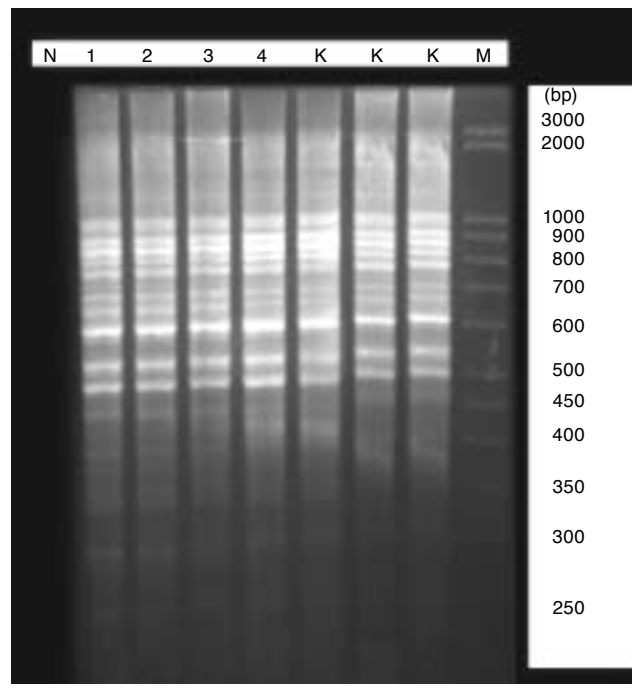

Figure 1. AP-PCR band patterns of test groups and control groups. Lanes 1-4 test groups; Lane 5-7(K,K,K) control groups; Lane 8 (M- Marker lane): 1kb step ladder (Fermantes)

Table 1. Literature regarding exposure and amount of nonviable microorganisms.

Author -Year	Exposure,	Time	Amount of nonviable microorganisms
Scherba et al.[16] 1991	1 W/cm^2, 24 kHz	2 dk	%22
	3 W/cm^2	30 dk	%39
Mason et al.[1] 2003	27 kHz	60 dk	%70
	27 kHz	5 gün	%85
Rediske et al.[3] 1999	300 mW/cm^2, 28.48 kHz	24 saat	3.61 $\log_{10}$
Current Study	30-161 mW/cm^2, 1.5 mHz	20 dk	%26.8

Discussion

In this study it was determined that application of LIPU for 20 min. cause significant alterations in bacterial count and morphology, whereas for remaining bacteria it is found that antibiotic susceptibility and genomic structure did not change.

Application of ultrasound waves for bactericidal effect and disinfection is a frequently using beneficial method especially in food, water sector and sterilisation of medical instruments.[2,15] The bactericidal effect of ultrasound waves is found to be related to multiple factors including width of bacterial wall, bacterial density in the area of application and duration, frequency and intensity of ultrasound process.[1,2,16] Bactericidal efficacy which was achieve by aplication sound waves in different studies was summarise in table 1. Disruption and stout appereance of the bacterial cellular wall in the test group on histological evaluation supports the knowledge of bactericidal effect of sound waves by diruption of cellular wall.[2] Although it seems paradoxical that cellular wall is thicker in destroyed bacteria than healthy ones, loosening after scattering of the cellular wall is the explanation of this finding.

Andrea M. Rediske et.al.[3] reported that treatment with low frequency (28.8-kHz), low intensity (100-300 mW/cm2) ultrasound, viable bacteria CFU/ml counts on biofilm were reduced for a total of 6.0 log10. When compared with antibiotherapy alone CFU/ml counts on biofilms treated with antibiotics and ultrasound demonstrated a further 2.39 log10 reduction in the same study. We showed that there was significant difference in viable counts between test and control groups. In the test groups while viable bacteria counts were reduced for a total of 5.0 log10, with further decrements of 3.66 log10 (p=0.000), complete eradication of CFU in the bacterial suspensions was never achieved. With this respect our study results were similar to reported findings of Rediske et al.[3]

It was shown that when ultrasound with higher intensity and duration was used, greater number of

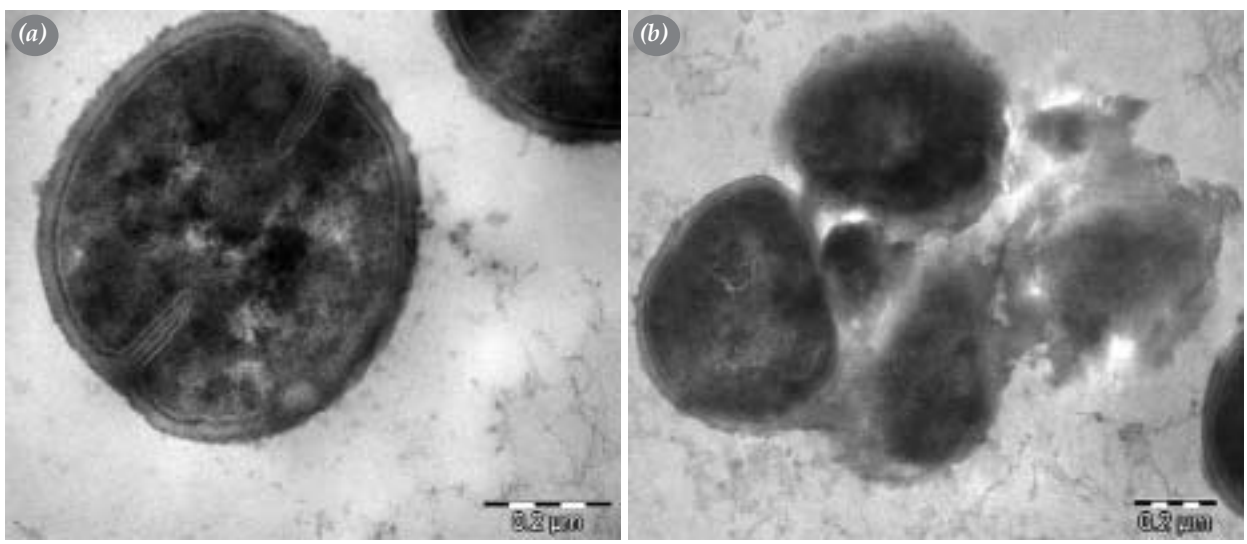

Figure 2. (a) From the control group. A dividing bacterium with normal morphologies (X 120.000). **(b)** From the test group. Some bacteria represent complete cell disruption in addition to the wall destruction (X 75.000)

Table 2. Literature regarding the effect of sound waves on DNA damage.

	Analysis System	Exposure, (Time)	DNA Damage
Cooter et al.[5] 2001	Human lymphocytes, Human chromosomes	20-50 kHz, 10-300 W/cm^2	Positive
Shintaku et al.[8] 1993	Mice fetal liver cells	2 MHz, 160.0-586.2 mW/cm^2 (10 dk)	Positive
Stella et al.[9] 1984	Human lymphocytes	0.860 MHz, 1 W/cm^2 (40-160 sn)	Positive/ Negative
Garaj-Vrhovac and Kopjar[6] 2005	Peripheral blood leucocytes	1-10 MHz, 0.05 W/cm^2	Positive
Miller et al.[7] 1989	Human leukocytes	1.45 MHz, 161 W/cm^2 (40-160 sn)	Positive
Takabayashi et al.[18] 1985	Mouse embrios	250, 500, 1000 Hz, >60 W/cm^2 (5 dk)	Positive
		250, 500, 1000 Hz, <60 W/cm^2 (5 dk)	Negative
Şahin et al.[22] 2004	Human lymphocytes	1 MHz (10 dk)	Negative
Miller et al.[23] 1991	Human lymphocytes	>1 MHz, 0.5-3 W/cm^2	Negative

bacteria was eradicated.[1,2,16] As might be expected, sonication of smaller volumes produced a more rapid kill.[17] Fatal effects on microorganisms and DNA damaging effect on animals of sonographically created were reported in the literature. This effect is explicit during continuous applications at > 58°C[18] For this purpose we monitorized heat by thermocouple in our study. But results of this monitorization revealed that there is no statistically significant difference between test and control groups. Despite our instrument was capable of producing higher frequency waves, heat generated did not reach to 60 degrees as dictated in the literature.[19]

Antimicrobial susceptibility test was performed by disc-diffusion method to viable bacteria which were processed by LIPU. There was no difference between test and control groups. Although its mechanism of effect is not known it is speculated that sound waves cause reduction in boundary layer thickness by mechanical factors like microconvection, cavitation events and turbulance with resultant enhancement in introduction of oxygen and antibiotics through membranes into biofilm.[20] There are also invivo studies in which proteolytic enzymes are tested for the infections related to implants and low antibiotic efficiency because of biofilm membrane.[21] Vollmer A. C. et al.[4] reported that higher frequency ultrasound stimulates specific stress response. It is reported in this study that sound waves cause changes on cellular wall. Electron microscopic findings of our study confirm these results.(Fig.2) However the intensity of sound waves needed for bacterial cell death or the amount of bubble charged for cellular destruction have not been determined yet. In addition, There is no consensus about the intensity, frequency and duration of sound waves which cause DNA damage.[4] DNA damage of sound waves aplications regarding datas in literature were summerised in table 2. Although DNA damage was shown in human lymphocytes and fetal liver cells in studies of some researchers[5], in which low frequency and low intensity (20-50 kHz, 10-300 W/cm2) and the other researchers[8] high frequency and low intensity (2 MHz, 160-586.2 mW/cm2) ultrasound were used respectively. Also the different studies[18,22,23] reported that there was no DNA damage when low frequency and low intensity (250-1000 kHz, 60W/cm2) ultrasound was used. There was no damage on bacterial DNA with high frequency and low intensity (1.5 mHz,30-161mW/cm2) ultrasound was used in our study.

We could not find any difference in genetic structure between test and control groups using AP-PCR analytical methods (Fig.1). However this technique is feasible in determination of genotipic differences between isolates, so advanced methods like DNA string analyse should give more detailed information about possible minor damage and basic changes in LIPU waves applied cells. Working on a single kind of bacteria and not employing the sound waves in different durations, frequency and intensities are the most important limitations of this study. The re-

sults after single application of sound waves in our study should be accounted as the findings of a preliminary invitro study, and sould be a guide for the investigations in which invivo efficacy of repeated sound wave applications are tested.

Low intensity pulsed ultrasound application can be thought as adjuvant modality against infections encountered in orthopaedic practice. As a result of in this study it is determined that application of LIPU by exogen device for 20 min. cause significant alterations in bacterial count and morphology, whereas for remaining viable bacteria it is found that antibiotic susceptibility and genomic structure do not change. In addition to this decrease of bacteria colony number via sound waves, could increase efficacy of antibiotics with indirect way.

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