Original

A Comparison of Six Ultrasound Stimulation Types on Pseudomonas Aeruginosa Growth in Vitro

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ABSTRACT

Background: This work evaluated the efficiency of common ultrasound stimulation (U.S.S) types on bacterial growth in vitro using clinically relevant conditions.

Objective: To estimate different frequencies ultrasound bactericidal ability on bacteria in bacteria of Pseudomonas Aeruginosa.

Material and Methods: Six types of U.S.S (continuous wave, 7w/cm^2, 20 KHz; continuous wave, 35w/0.8L, 40 KHz; continuous wave, 5w/cm^2, 1.1 MHz; pulsed wave, 5w/cm^2, 3.3 MHz; continuous wave, 5w/cm^2, 3.3 MHz and continuous wave, 0.5w/cm^2, 3.5 MHz) were applied to a separate set of culture plates containing *Pseudomonas aeruginosa* for 10 minutes at room temperature on four sample sets to inhibit bacterial growth. After US.S treatment, the zone of inhibition at the US probe location was measured.

Results: Zone of inhibition measurements demonstrated a significant inhibitory effect for continuous wave US.S of 5w/cm^2, 1.1 MHz; pulsed wave US.S of 5w/cm^2, 3.3 MHz; and continuous wave US.S of 5w/cm^2, 3.3 MHz ($p < 0.05$), but not for continuous wave US.S of 7w/cm^2, 20 KHz; continuous wave US.S of 35w/0.8L, 40 KHz; and continuous wave US.S of 0.5w/cm^2, 3.5 MHz.

Conclusion: The data suggest that for infected wounds, continuous wave US.S of 5w/cm^2 and 1.1 MHz; pulsed wave US.S of 5w/cm^2 and 3.3 MHz; and continuous wave US.S of 5w/cm^2 and 3.3 MHz ultrasound treatments may have an initial bacterial inhibitory effect, which does not significantly change with subsequent treatments.

Keywords

Bacteria, Pseudomonas Aeruginosa, Bactericidal, Inhibition, Ultrasound Stimulation, Wound Healing

Introduction

Different types of ultrasound stimulation (US.S) have been used as a new strategy to inhibit microorganisms in various aqueous environments [1]. Possible mechanisms which may account for enhanced wound healing include bactericidal and bacteriostatic effects include sonochemical interactions produced by acoustic cavitations and mechanical effects [1]. The effect of US.S on wound healing has been examined in a number of research studies which checked low and high frequency pulsed and continuous ultrasound waves, all show-

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ing evidence of decreased wound healing time using US.S [2]. As known bacteria represent a pathogenic problem that cause a lot of dangerous diseases and scientist continuously develop new drugs to resist pathogenic bacteria and safe patient's life, a lot of bacterial types due to their nature have the ability to develop an antibiotic resistance using their virulence factors [3, 4]. A study by Yadollahpour et al. shows that US.S can accelerate wound healing of chronic wounds, purulent wounds, trophic ulcers, pressure ulcer and extremity lower wound [5]. Any acceleration of chronic ulcer closure would naturally result in economic savings and decrease in amputation rates. Accordingly, basic evidence has been established to support US.S role in wounds treatment which have not responded to conventional therapy [5].

Many trails to estimate the effects of US.S on bacteria usually found in wounds have resulted in the publication of several in-vitro works. These works examined various US.S types including ultrasound of 10 mW/cm^2 ultrasound at frequencies of 70 kHz, 500 kHz, 2.25 MHz and 10 MHz [6], ultrasound of low-kilohertz range (20 and 38 kHz)[7, 8] and higher frequencies ranging (512 and 850 kHz) [7], ultrasound frequency of 1.1 MHz creating peak compressional and rare factional pressures at the disk surface of 30 and 13 MPa [9], and ultrasound of an intensity of 2.5 W/cm^2 and frequency of 40 kHz[10].

The US.S sonochemical interactions caused by the formation, growth and implosive collapse of bubbles in a liquid during which an intense heating of the bubbles occurs, leads to hot spots temperatures of closely 5000°C, a pressures of closely 500 atmospheres and lifetimes of a few microseconds. Shock waves from cavitation in liquid-solid slurries produce high-velocity antiparticle collisions, the impact of which is sufficient to melt most metals through the process called acoustic cavitations [11, 12]. This is while, US.S mechanical effects produce high pressure changes in tissues leading to various effects from target vibration to cavitation [13].

Higher results of US.S will take place at a specific resonance frequency which represents object natural frequency causing the well-known event of Tacoma Narrows's bridge failure in 1940 [14]. Resonance means that the energy which the oscillator absorbs and dissipates as a function of the excitation frequency as a maximum value [15, 16].

$$fo = \frac{1}{2\delta}\sqrt[2]{\frac{k}{m}} \qquad (1)$$

Much scientific research has examined ultrasound on organisms at the beginning over short periods of time. Sonication effects on non-target organisms might be more than effects confirmed in short-term laboratory studies if anti-algal units are employed continuously as encouraged by manufacturers. Acknowledgements on the efficient wavelengths and intensities used by the devices, however, are proprietary and publicly unauthorized. Therefore, dependent studies, those using ultrasound frequencies, are thought to be similar to those of anti-algal, sonication units on non-cyanobacterial organisms [17].

This study examined a pathogenic agent, Pseudomonas Aeruginosa, to estimate the most effective frequency on this bacterium to inhibit its growth.

Material and Methods

Materials

1. Medical US device, physiotherapist unit (Supreme Surgical Co. /Indian made)
2. Medical Diagnostic US device (Ccontic TM CMS 600B-2/ Chinese made)
3. Skin Cleaner (LW-006) Chinese
4. Ultrasonic cleaner (Shenzhen, SKYMEN, JP-008) Chinese
5. Colours photon & Ultrasonic skin cleaner (Beauty instrument, LW-013) Chinese
6. US gel

7. Trypticase Soya Broth (**TSB**)
8. Agarose
9. Phosphate Buffer Slain (**PBS**)
10. Cetrimide agar.

Method

1- A 6 gm of TSB powder (concentration 30 gm/L) was dissolved in 200 ml of distilled water, sterilized at 121ºC for 15 minutes, the broth cooled at room temperature, 1 ml from PA stock (2.4×10^8 cfu/ml), poured in the broth and mixed gently by hand. Finally, the cultured broth incubated at 37ºC and 200 rpm for 20-22 hours using shaker incubator.

2- A 4.93 gm of PBS (concentration of 9.86gm/L) was dissolved in 500ml of distilled water and mixed manually, adding 1.5 gm of agarose to 150 ml of the prepared PBS solution and mixed well manually. Then, it was completely dissolved and sterilized using microwave for about 140 sec. After cooling to about 37ºC at room temperature, 10% (i.e. 15 ml) of activated PA by TSB was added to the mixture and mixed gently. Finally, the cultured agar was poured in 60mm petri dishes and cooled at room temperature for solidification.

3- Petri dishes containing cultured PA were stimulated directly using ultrasound by locating US probe on the base (bottom) of the petri dish via US gel as shown in Figure 1; then the stimulated PA was incubated at 37ºC for 20-22 hours to observe and measure the inhibition zone. This operation was repeated for all listed US machines.

4- PA cultured petri dish was placed in a water bath, then the probe was placed in the same water container and the stimulation started by US for a specific time as shown in Figure 2, after that, the sample was incubated at 37ºC for 20-22 hours. Finally, inhibition zone was observed, measured and recorded.

5- The inhibition zone was measured using a roller.

6- A smear from the inhibited and non-inhi ited zones was cultured in petri dish containing cetrimide agar.

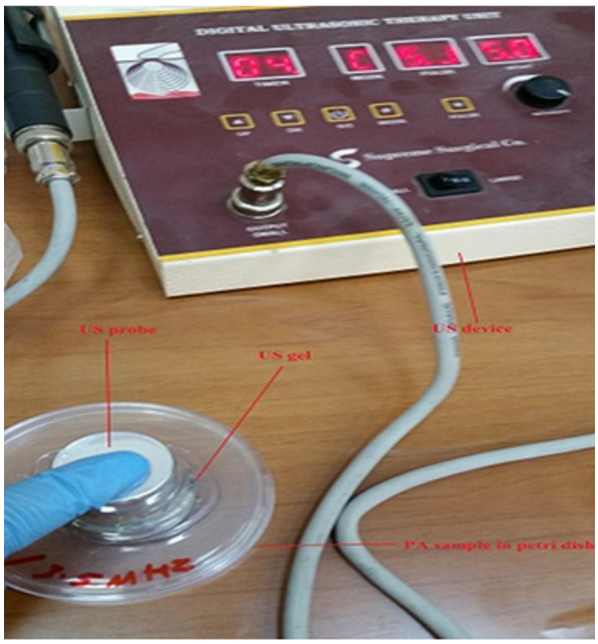

Figure 1: PA cultured petri dish stimulated directly by US physiotherapy machine

Results

After the end of stimulation and incubation for 20-22 hours, the inhibition zones (if found) were measured and the results were recorded as shown in Table1and the related Figures.

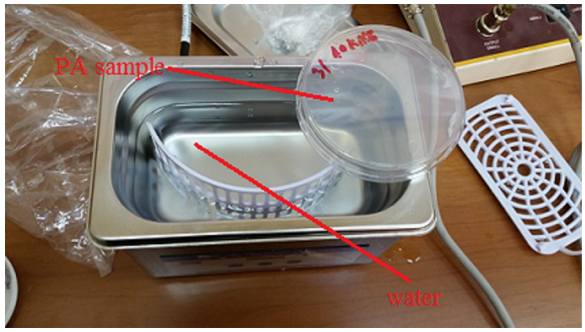

Figure 2: PA cultured petri dish stimulated by US machine through water

Discussion

This section of the study aimed to prove that low energy of US could cause bactericidal effect on PA. From the results of US stimulation inhibition zone, it was detected that when the CW, 5w/cm², 1.1 MHz, 5 pulses and the stimu-

Table 1: inhibition zone diameter of the US stimulated petri dishes.

No.	Date 2017	US Frequency, Hz/ Energy, w/cm²	Stimulation time, minutes	Inhibition zone, mm	Notes and related figure
1.	11/4	CW, 7w/cm² 20 KHz	10	0	Direct stimulation Figure 3/ sample 3
2.	11/4	CW, 7w/cm² 20 KHz	10	0	Through waterPoor growth Figure 3/ sample 4
3.	19/1	CW, 7w/cm² 20 KHz	10 &5pulse	20	Direct stimulation Figure 4/ sample 1
4.	19/1	CW, 7w/cm² 20 KHz	15 & 5pulse	20	Direct stimulation Figure 5/sample 3, not clear view because US gel effect.
5.	11/4	CW, 7w/cm² 20 KHz	10 & 5pulse	15	Direct stimulation Figure 3 and 6 / sample 1
6.	11/4	CW, 7w/cm² 20 KHz	10	0	Direct stimulation Figure 3
7.	19/1	CW, 7w/cm² 20 KHz	10 &5pulse	5	Direct stimulation Figure 4/sample 2, not clear view because US gel effect.
8.	11/4	CW, 7w/cm² 20 KHz	10 & 5pulse	6	Direct stimulation Figure 3 and 6 / sample 2
9.	19/1	CW, 7w/cm² 20 KHz	10	0	Direct stimulation Figure 5/sample 4

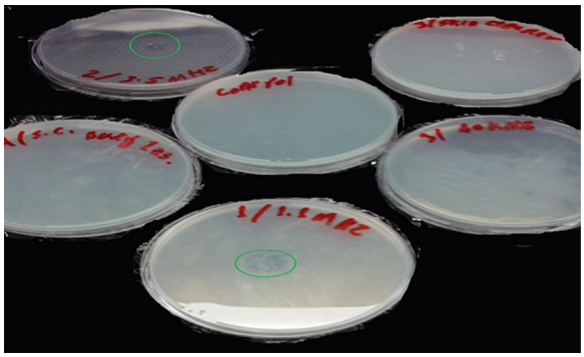

Figure 3: US effect direct on petri dish at 20 KHz, 40 KHz, 1.1 MHz, 3.3 MHz for 10 min

lation time for 10 minutes, the reading of the inhibition zone diameter was 20 mm via direct stimulation as in figure (4.29). It was the effective dose to inhibit PA growth significantly. When the frequency was 3.3 MHz, the inhibition zone diameter was 6 mm as in figure (4.24) in comparison with other values of US doses (which shows no inhibitory effect) and the control group. It is statistically clear that US effect in ranges of physiotherapist energy at frequency of 1.1 MHz and 3.3 MHz demonstrates maximum inhibition effect on PA bio-

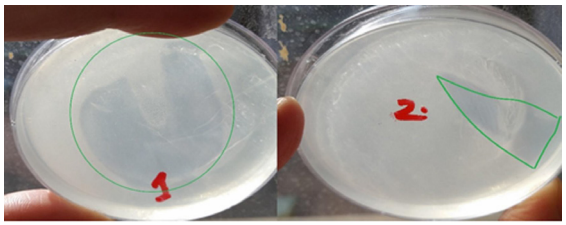

Figure 4: Ultrasound effect using 1.1 MHz & 3.3MHz and gel

film growth which is in agreement with Jin Xu et al. [2012] who found that 1.1 MHz US of pulses equal to 20 cycles in duration caused peak compressional and rarefactional pres-

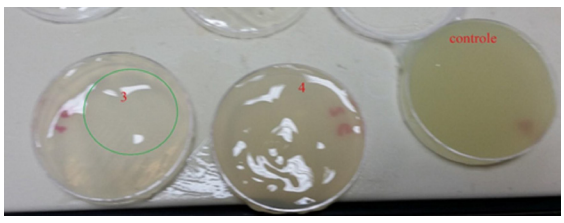

Figure 5: Ultrasound effect, direct petri dish stimulation using gel at 1.1MHz and 3.5MHz for 15 and 10 min. respectively

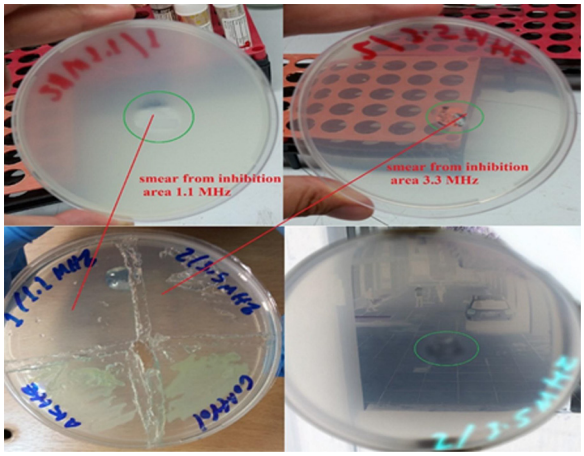

Figure 6: Ultrasound effect, direct stimulation on petri dish at 1.1 MHz and 3.3MHz for 10 min. Also a smear from inhibition zone culturing is shown

sures at the disk surface of 30 and 13 MPa, respectively. PAs were tagged with green fluorescent protein (GFP) as well as cells killed by high-intensity focused ultrasound (HIFU) were visualized using propidium iodide, which permeates membranes of dead cells [9].This, however, means that local Iraqi PA isolation has a common resonance property the same as other isolations from the USA so the destruction happens because of some vital contents of PA included in both isolations. This inhibitory effect reduced at frequency of 3.3 MHz, while low energies generated by US diagnostic machine show no inhibitory effect using very close frequency (3.5 MHz) which might be due to its low energy or that 1.1 MHz was a natural frequency of PA or vital PA content; 3.3 MHz is its 3rd harmonic frequency while 3.5 MHz is not related to natural frequency 1.1 MHz. Frequencies valued as 20 KHz and 40 KHz with high energy are widely used in cell disrupter machine, while in the present study they were not effective for PA growth inhibition because used energies were low.

These results reveal that US energy, frequency and time are all significant factors but resonance frequencies have the advantage of killing microorganisms at relatively low energy safe to human body.

Conclusion

This study proved that low values of mechanical waves, in range of US which are characterized by clinically accepted energies and stimulation times, could inhibit the growth of microorganisms specifically for Pseudomonas Aeruginosa. It was shown that US could inhibit PA growth, so physical ultrasound effects can be developed to be used as a therapeutic technique to treat PA or other microorganisms because they show statistically significant inhibitory effects on PA growth using clinically suitable stimulation energy and time.

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Conflict of Interest

None.

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