Free hydroxyl radical dosimetry by using 1 MHz low level ultrasound waves

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INTRODUCTION

In recent years the use of low output intensity ultrasound in therapeutic medicine is a developing field. The main fields of study are in sonodynamic therapy (SDT), improving chemotherapy and gene and apoptosis therapy and drug delivery (1, 2, 3). These new methods of therapy have great potential because of their relatively easy application(1). Although the mechanism of low output intensity ultrasound in treatment malignant tissues are not well understood, however, the non-thermal effects of ultrasound, especially cavitations are considered to be a primary reason for these purposes (4, 5, 6).

Background:

In order to quantify effects of ultrasound irradiation parameters under therapeutic condition, especially sonodynamic therapy, it is initially necessary to evaluate inertial cavitation activity in vitro conditions; therefore, in this study, the effect of 1 MHz low level ultrasound based on °OH radicals generated by acoustic inertial cavitation in aqueous solution was monitored by their reaction with terephthalic acid (TA) to produce fluorescent 2-Hydroxycinate acid (HTA) by spectrofluorometry method (Terephthalic acid dosimetry). Materials and Methods: The study was designed to measure hydroxyl radicals in a field near to 1 MHz sonotherapy probe in progressive mode and low level intensity. The effect of ultrasound irradiation parameters (1MHz) containing duty factor, mode, intensity ultrasound and, time sonication in hydroxyl radical production have been considered. After preparation of solution of dosimetry and plotting calibration curve of spectrofluorescence, the effect of mode of sonication (continuous and pulsating), duty factor (20-80%), intensity (0-2 W/cm², with step of 0.5 W/cm²), and sonication time (0-60min with step time of 10min) without increasing temperature to more than 3°C to determine the effective exposure in low level ultrasound were evaluated. The fluorescence intensity of TA solution before and after irradiation, in all cases was measured, and the results were reported as Mean ± Standard Deviation (SD). Results: The result of experiments related to sonication mode for 1MHz ultrasound irradiation (2 W/cm²) show that continuous mode of sonication is 29% higher fluorescence intensity than the pulse mode in 80% duty cycle for sonodynamic therapy. With compensation of irradiation time for 1MHz sonication in different duty cycles, fluorescence intensity in continuous mode is 22% higher than the pulse mode in average. The amount of hydroxyl radicals production versus ultrasound intensity, and sonication time show with increasing intensity or sonication time in continuous mode, the hydroxyl radical production is linearity increased (R=0.99). Conclusion: The results show that the terephthalic acid dosimetry is suitable for detecting and quantifying free hydroxyl radical as a criterion of inertial cavitation production over a range of condition in medical ultrasound fields.

Keywords: Low level ultrasound, cavitation, terephthalic acid dosimetry, free hydroxyl radical dosimetry

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evaluate inertial cavitation activity in vitro conditions. This is very important because it has been prompted by safety in considerations and also with a view of applied low output intensity ultrasound therapy in therapeutic conditions.

It was previously shown that the chemical dosimeters are suitable for ionization radiation dosimetry and also for detection and quantifying free radicals generated by collapse of cavitation in medical ultrasound fields (12, 13, 14). These methods use aqueous solutions, which on reaction with free radicals formed during water sonolysis, forms specific compounds which are readily detected by using spectroscopy methods. Simplified equations for production of radicals by collapse of cavitation in water solution are shown in equations 1 to 4.

### MATERIALS AND METHODS

#### Dosimetry solution

The dosimetry solution of terephthalic acid was prepared according to the standard protocols; containing terephthalic acid (2×10³ mol/L (Aldrich) dissolving with heating, NaOH 0.200g (5×10³ mol/L) and phosphate buffer (pH 7.4), prepared from KH₂PO₄ (4.4×10³ mol/L, Merck) and Na₂HPO₄ (7.6×10³ mol/L, Merck). The resulting solution was then made up to 1dm³ with distilled and deionized water. Before use, the solution was kept in refrigerator (~4°C) and in dark condition to prevent photochemical reaction. Periodically, during sonication samples were removed and the fluorescence intensity (counts) for samples measured using a spectrofluorometer (Shimatzo, Model RF-1500, Japan) with an excitation and emission wavelengths of 310and 425.6 nm, respectively. To accelerate the final reaction product (2-hydroxyterephthalic acid), and to increase fluorescence intensity efficiency and simple measurements, IrCl₆⁻ (2×10⁴ mol/L, Aldrich) was used as a potent oxidant, in dosimetry solution (14, 15, 16). After preparation of dosimetry solution the sonication of samples in all experiments was performed in constant concentration with respect to TA (2×10³ mol). In experiments the irradiated TA solution was kept in darkness and their fluorescence intensities were measured within 2-4 hr after sonication.

#### Calibration curve of spectrofluorescence

Initially 2-hydroxyterephthalic acid was synthesized and a stock solution of the standard 2-hydroxyterephthalic acid (HTA) was prepared by reaction of bromoterephthalic acid with sodium hydroxide (11). Volumes of solution of 500 cm³ were made up with water. The concentration of the solution with respect to HTA was 2×10⁵ mol/L. The stock solution was diluted initially to a concentration of 2×10⁴ mol/L (1cm³ to 100cm³) and then using this solution further dilution of known concentration were made. The fluorescence of each solution was measured using spectrofluorometer with an excitation and emission wavelengths of 310 and...
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425.6nm, respectively. Fluorescence intensity versus HTA concentration was plotted and given a straight line of positive slope for concentration from $1 \times 10^6$ to $1 \times 10^5$ mol/L.

Hence, it can be assumed that the fluorescence intensity (counts) generated by sonication of samples is proportional to the concentration of the HTA formed in the samples and this, in turn, a measure of the quantity of °OH radicals formed by inertial cavitation activities. So, the inertial cavitation activity indirectly is proportional to the fluorescence intensity (counts).

Equipment setup

The applied ultrasound source (T) was a 1MHz apparatus (Sonoplus 462, Enrof Nonius Co., Netherland) with a Piezoelectric transducer probe 30mm of diameter and 5cm² effective radiation area. Acoustic calibration for power and intensity of this device was carried out in degassed water by using an ultrasound power meter balance, model UPM-DT-10 (Netech, USA) with limit of uncertainty ±1mW. All intensities quoted are spatial average temporal average (SATA) in experiments. By this apparatus, we were able to change mode of sonication (continuous and pulsating), Duty factor (20-80%), intensity (0-2W/cm², with step of 0.5W/cm²) and sonication time (0-60min with step time of 10min) without increasing temperature more than 3°C.

Irradiation condition

For exposure under controlled conditions, a cubical insonation chamber for irradiation of dosimetry solution with a volume of 7cm³ was constructed from PVC with their surfaces membranes made from ‘Parafilm’ which is a thin sound transmission film and located in near fields of probes at 5mm away from surface of probes in vertically position. It was placed in same position in all experiments by a clamp and rotate axis which allowed precise adjustment of position in its location. This chamber containing sample solution that was put in cubical tank in dimensions of 25×10×10 cm³ which was constructed from polyacrylic material and filled with degassed water. To eliminate the production of air bubbles between the probes and the TA chamber, a small amount of detergent was added to the tank. To perform experiments under progressive-wave conditions and limit action of acoustic reflection from the wall of tank, the inner surfaces of the front of probe was covered by absorb ultrasound material. The temperature of the solution in the chamber was monitored during insonation and no change of greater than 2-5 °C was noted. The probe of ultrasound was fixed in the tank wall and ultrasound beam was emitted vertically into the TA chamber, located at a 5mm of from the probe.

Fluorescence spectrum

Using terephthalic acid dosimetry method, it was possible to evaluate the effected efficacies of irradiation parameters on the inertial cavitation activity in medical ultrasound fields by monitoring OH radical production (fluorescence count). This is an indirect method for estimation of inertial cavitation activity in ultrasound fields. The ultrasound irradiation parameters that have been studied consist of the mode of sonication, duty cycle, intensity and, sonication time and or dosage "Surface Energy Density (SED)". SED in experiments is defined as:

\[
\text{SED (J/cm}^2) = I \text{(W/cm}^2) \times t \text{ (Sec)} \times \text{D.F} \text{ (%Duty factor)}
\]

The fluorescence intensity of TA solution before and after irradiation, in all cases was measured and the results were reported as Mean ± Standard Deviation (SD).

RESULTS

Sonication mode for 1MHz ultrasound irradiation

Result of the experiments related to sonication mode for 1 MHz ultrasound irradiation is shown in figure 1. As seen, the amount of fluorescence intensity obtained using 1 MHz ultrasound in continuous mode is significantly more than the pulse mode with different duty cycles (pair t-test analysis with p-value <0.05).
Results indicated that any increase in duty cycle will be associated with an increase in fluorescence intensity and, the fluorescence intensity in continuous mode of sonication is higher than the pulse mode in different duty cycles. The time of sonication in different duty cycle is not equal hence producing different fluorescence intensity. Therefore, we have irradiation time for 1MHz sonication in different duty cycles is compensated (figure 2).

From figure 3, it can be seen that with compensated time of irradiation in various duty cycles the fluorescence intensity approximately reach to continuous mode. This is the results of the exposure time in constant intensity or dosage of ultrasound: Therefore, if SED in the field of ultrasound irradiation is constant, the difference between amount of fluorescence intensity in different duty cycles will not be significant (p-value>0.05).

For compensated intensity irradiation, experiments were performed at different duty cycles with compensated intensity irradiation. In this study, the time of irradiation was constant and the sound intensity was increased during experiment gradually. It can be seen that the fluorescence intensity in all cases are approximately equal (figure 3). During experiments the SED was constant for different duty cycle, thus, the difference between the amounts of fluorescence intensity is not significant.

**Effect of sonication intensity on fluorescence intensity**

As expected, the inertial cavitation activity increased at higher intensity (9, 11, 12, 17) and, in this regard, it is instructive to consider the rate of increase on the amount of fluorescence intensity for 1 MHz ultrasound therapy.
system. For this purpose, by selecting continuous mode of irradiation, we described the effect of intensity irradiation on cavitation activity using this mode of sonication. A series of 20 min exposure were carried out and during experiment the intensity in low level range was gradually increased (0.5, 1, 1.5 and 2 W/cm²), and the dose response of ultrasound, versus the amount of fluorescence intensity has been considered (figure 4).

Results show that the correlation between intensity irradiation in low level intensity, versus fluorescence intensity, is linear (R=0.998, Correlation is significant at the 0.01 level). So, the efficiency of formation of 2-hydroxyterephthalic acid (HTA) in sample solution is proportional to OH radical production, in low level intensity irradiation is linear versus amount of fluorescence intensity which, this is a criterion of acoustic cavitation production in ultrasound fields.

**Sonication time**

It is well known that the amount of fluorescence intensity for long time irradiation is increased (9, 11, 12). In this regard, instructive to the amount of fluorescence intensity for 1 MHz ultrasound therapy system in continuous mode of sonication. On the other hand, since the sonodynamic therapy method is down in temperatures under the threshold of temperature in which the hyperthermia effects in biological environments is appeared, therefore, in addition the role of irradiation time on the amount of fluorescence intensity, it is important to clear the rate of increase temperature inside the sample solution for long time exposure. For investigation this phenomenon, the 1 MHz ultrasound irradiation with continuous mode was selected (1 MHz con) and the action of irradiation carried out in exposure times of 10-50 minutes with steps of 10 minutes. The experimental results are shown in figure 5.

Figure 5 shows the time of irradiation is effective in increasing on fluorescence intensity and temperature of medium in TA solution. The correlation coefficient between sonication time in low level intensity, versus amount of fluorescence intensity, is linear (R=0.995, Correlation is significant at the 0.01 level). Despite the increasing temperature in medium of TA solution for long, the exposure (60 min), it is still under the threshold of hyperthermia affecting environments.

**DISCUSSION**

There is considerable interest in the evaluation of inertial cavitation for possibility of sonodynamic therapy which uses low level
ultrasound to release or enhance the action of therapeutic agents in vivo. In order to quantify inertial cavitation, certain methods are necessary for determining and quantifying inertial cavitation which are widely applicable methods. Existing methods for performing these experiments including acoustic imaging, sonoluminescence and other methods for example the electron spin resonance (ESR) or laser holography (12) are difficult and expensive. The electron spin resonance (ESR) is an extremely sensitive method for detecting the radical produced but its application needs specialist and expensive equipment (12, 14). Other methods to monitor the chemical effects of inertial cavitation and free radicals produced are suitable to detect radical species (11, 12, 14, 17). Currently, cancer targeted therapy with low level ultrasound, sonodynamic therapy, is introduced. In this study, the collapse of cavities can produce interesting chemical effects, known as sonochemistry (14). In order to develop effective sonodynamic therapy, evaluation of effective parameters is the necessary concluding type of ultrasound mode, duty factor, intensity, time with and without constant surface energy density; the method was developed for in vitro use.

In this study hydroxyl radical production is measured in the field of 1 MHz ultrasound waves at low level intensity by terephthalic acid dosimetry method. This method of dosimetry is specific for trapping of hydroxyl radical production in fields of ultrasound irradiation because each molecule of hydroxyl radical is trapping by one molecule of terephthalic acid and formation one molecule of 2-Hydroxyterephthalate acid (HTA) that can be measured by fluorescence spectroscopy method. Because this method of dosimetry is based on fluorometry method, it is very sensitive for hydroxyl radical measurement. On the other hand, the ultrasound irradiation parameters such as duty factor, mode of sonication, intensity and time of irradiation, also, surface energy density (SED) are effective in hydroxyl radical production and in turn, in inertial cavitation production. In equal SED for every irradiation mode, the amount of fluorescence intensity approximately is constant. This is due to dosage of energy release by ultrasound irradiation.

Since the sonodynamic therapy is performed at temperatures under the threshold of hyperthermia effect on biological environment therefore, in all experiments, temperature changes must be less than 2-5 °C. The results of experiments related to sonication mode for 1 MHz ultrasound irradiation (2 W/cm²) show that the continuous mode of sonication is 29% higher fluorescence intensity than the pulse mode in 80% duty cycle for sonodynamic therapy. With compensation of irradiation time for 1 MHz sonication in different duty cycles, approximately, fluorescence intensity in continuous mode is in average 22% higher than the pulse mode.

The amount of hydroxyl radicals production versus ultrasound intensity and sonication time show that, with increasing intensity or sonication time in continuous mode, the hydroxyl radical production is linearity increased (R=0.99). By determination of these parameters, it is possible to control inertial cavitation in low level fields of high frequency ultrasound waves for effective sonodynamic therapy.

CONCLUSION

Terephthalic acid dosimetry is a suitable method for monitoring the acoustic cavitation effects by measurement of hydroxyl radicals in medical ultrasound ranger. The use of fluorescence intensity provides a measure of the efficiency of production of hydroxyl radicals via ultrasound irradiation. It should be remembered, however, that this method employs a chemical dosimeter and as such it may be only, the ideal dosimeter for estimation of inertial cavitation production. On the other hand, the ultrasound irradiation parameters such as duty factor, mode of sonication, intensity and time radiation and SED are effective in hydroxyl radical production and of turn, in inertial cavitation production. For various duty cycles, the fluorescence intensity increases at higher duty cycle (80%>50%>20%), also in continuous mode of
sonication the amount of fluorescence intensity is higher than the pulsed mode. The inertial cavitation activity in higher dosage is more than the low dosage.

The temperature in medium of TA solution for long exposure time (60 min) is increased but it is still under the threshold of temperature affecting biological environments (T < 40°C). Therefore, by control of these irradiation parameters in ultrasound fields, it is possible to control inertial cavitation. Although, the inertial cavitation activity in in vitro conditions is different respect to in vivo condition, but it is believed that the investigation of ultrasound irradiation at low level intensity can be useful for medical therapeutic purposes, especially for superficial tumors treatment.

ACKNOWLEDGEMENT

The authors greatly appreciated the help of Professor Timothy J. Mason in Coventry University for preparation and synthesis of standard solution of 2-hydroxyterephthalic acid.

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