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SCIENTIFIC PAPER

A comparative study on generating hydroxyl radicals by single and two-frequency ultrasound with gold nanoparticles and protoporphyrin IX

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Abstract

Sonodynamic therapy (SDT) is a new manner of killing cancer cells based on the cytotoxic interactions of ultrasound with sonosensitizing agents. It is shown that gold nanoparticles (GNPs) increase the efficiency of cavitation activity of ultrasound. In this study the influence of a single and/or two frequencies of ultrasound waves to generate hydroxyl radicals ($^{\cdot}$OH*)* was assessed in the presence of protoporphyrin IX (PpIX) and/or GNPs. Ultrasound cavitation activity was determined by recording fluorescence signals from chemical terephthalic acid (TA) dosimeters with or without PpIX and/or GNPs at the frequencies of 0.8 and 2.4 MHz individually and aggregately. To study hydroxyl radicals, experiments were performed with and without hydroxyl radical scavengers mannitol, histidine, and sodium azide. Cavitation activity was amplified by increasing ultrasound intensity and exposure time. The cavitation activity induced by dual ultrasound frequency was remarkably higher than the summation of effects produced by individual frequencies. All three scavengers reduced the fluorescence signal level. The effect of GNPs on intensifying cavitation activity at higher frequency was greater than that of lower frequency. PpIX showed a more effective sonosensitizing property at the lower frequency. Also, estimated synergism at dual frequency irradiation was improved in the presence of GNPs. We found that GNPs increased hydroxyl radical production at 2.4 MHz and that PpIX increased hydroxyl radical production at 0.8 MHz. Dual frequency exposure was more effective than single frequency exposure. PpIX at low frequency and gold nanoparticles at high frequency both enhance sonodynamic treatment efficacy.

Keywords Sonodynamic therapy · Protoporphyrin IX · Gold nanoparticles · Terephthalic acid · Hydroxyl radicals · Ultrasound · Cavitation

Introduction

Sonodynamic therapy (SDT) is an anticancer therapeutic approach based on the cytotoxic interaction of ultrasound and certain chemical compounds called "sonosensitizers". In SDT, tumor cells are killed by ultrasound in the presence of hematoporphyrin derivatives [1]. Therapeutic effects can be mediated by free radicals, chiefly singlet oxygen and hydroxyl radicals [2–4]. Ultrasound can focus on a small region of target which can deeply penetrate tissue, so SDT may be a useful tool for the clinical treatment of tumors [5].

High intensity focused ultrasound (HIFU) functions through both thermal and mechanical mechanisms [6]. Sonodynamic therapy is the outcome of the non-thermal effect of ultrasound, especially cavitation [7–14]. Mechanical effects of HIFU are cavitation, radiation force, shear stress, and acoustic cavitation is related to the formation. Cavitation is related to the formation and activity of a gas

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filled bubble under acoustic excitation in a medium [15]. The acoustic cavitation threshold depends on a number of factors, such as cavitation bubble size, power, surrounding medium properties, and ultrasound frequency [16–19]. Experimental findings have shown that cavitation activity caused by multi-frequency ultrasonic irradiation is greater than that of single-frequency radiation, even at a low intensity [2, 20–28].

The mechanism of multi-frequency excitation for enhancing the inertial cavitation is still not exactly known, however several studies have been devoted to understanding the effect [29]. Matsumoto et al. showed that the acoustic cavitation induced by ultrasound is strongly dependent on ultrasound frequency [30]. Although most studies detail only the enhancement of dual- or multi-frequency fields, there have been some reports on the inhibition that can be observed under certain conditions [19]. Dezhkunov observed that the sonoluminescence decreases at high acoustic intensity with dual frequency sonication. This effect was attributed to an over-saturation of bubbles in the cavitation zone [19, 31].

Protoporphyrin IX (PpIX) is an efficient photosensitizer and sonosensitizer activated by light and ultrasound waves [32]. The interaction of activated PpIX with molecular oxygen produces reactive oxygen species (ROS), which can lead to irreversible cell destruction [10]. Recently, gold nanoparticles (GNPs) have been used in cancer therapy due to their special optical properties [33, 34]**.** A few studies have shown some sonosensitizing characteristics of nanoparticles where they enhance the thermal and mechanical interactions of ultrasound with tissues [35]. Vincent et al. reported that the presence of magnetic nanoparticles increases the amount of spheroid disintegration and enhance the efficacy of HIFU in destruction of the tumorous tissues [36]. Low toxicity and anti-angiogenesis properties make GNPs highly efficient in medical applications [37, 38].

On the other hand, the non-radiative relaxation time of PpIX in the presence of GNPs is longer than in their absence [39]. In this paper, the ultrasound cavitation activity is compared in single and dual frequency harmonics of 0.8 and 2.4 MHz. Also, the role of PpIX and/or GNPs in intensifying or reducing cavitation activity after irradiation by these ultrasonic frequencies in the aqueous media is investigated.

There are various methods for detecting cavitation activity based on measuring free radical concentration and chemical reaction products [40–42]. The suitability of terephthalic acid (TA) for detecting and quantifying free hydroxyl radicals generated by the collapse of cavitation bubbles in ultrasound fields has been shown [40–43]. In the present study, TA solution, as a chemical dosimeter has been adopted to evaluate ultrasonic cavitation activity in the presence of PpIX and/or GNPs after irradiation of TA solutions by single and dual frequency ultrasound at therapeutic intensities. Many SDT studies have shown that ROS generation can result in cell death [44–48]. Wang et al. used PpIX as a sonosensitizer. They demonstrated obvious ROS formation immediately after SDT application [49].

This study examines the effects of GNPs and/or PpIX on changing the sonodynamic process. Its findings would help optimize the synthesis of conjugated GNP/PpIX. The goal of using GNPs is to answer the question of whether GNPs can increase PpIXsonodynamic activity. Other goals of this research follow the effect of single and/or two-frequencies patterns of ultrasound exposure with different intensities on the efficiency of $^{\cdot}$OH generation in the presence of PpIX and GNPs. Also, the role of combining two harmonics of ultrasound in the presence and absence of GNPs and/or PpIX in generating hydroxyl radicals were investigated.

Materials and methods

Reagents

The 15 nm GNPs were prepared in the School of Pharmacy, Mashhad University of Medical Sciences, Iran [38, 48].

Protoporphyrin IX (Sigma-Aldrich, Germany) was dissolved in Dimethyl sulfoxide (DMSO) (1 mg PpIX was dissolved in 0.05 ml DMSO and 9.95 ml distilled deionized water; 0.178 mM). Protoporphyrin IX was used with concentration of 1 µg/ml. Terephthalic acid (Aldrich, Germany) was used to prepare chemical dosimeters. Mannitol (Molecular weight = 182.17 g/mol), histidine (Molecular weight = 155.16 g/mol, $C_6H_9N_3O_2$) and sodium azide (Molecular weight = 65.01 g/mol, NaN_3) were purchased from Sigma Company. Three scavengers were used with concentration of 10 mM.

Instruments

Dosimeters were irradiated by an ultrasonic therapy device (Ultrasound Sonozimer Co., Germany) equipped with two ultrasonic flat transducers with the area of 5 and 1 cm^2 (in this study, the applicator with 5 cm^2 area was used) in continuous and pulsed (with duty cycle of 50%) modes at different frequencies of 0.8 and 2.4 MHz, simultaneously and separately. After ultrasound exposure to different TA samples, the fluorescence signal was recorded using a spectrofluorimeter (FP-6200, Jasco, Japan) with excitation and emission wavelengths of 310 and 420 nm, respectively.

Chemical dosimeters and experimental groups

The performance of ultrasonic irradiation for cavitation activity was assessed by monitoring OH radicals produced in TA dosimeters [40, 43]. TA is a non-fluorescent molecule, in this method, TA solution, as a chemical dosimeter, reacts with a hydroxyl radical generated by water sonolysis with the resultant 2-hydroxy terephthalic acid (HTA) being detectable by a spectrofluorometer. The dosimetric solution was prepared at a concentration of 2×10^{-3} mol TA dissolved in 1000 ml phosphate buffer solution (PBS) while heated [43]. Fluorescence intensities of dosimeters were measured after ultrasound irradiation. The conditions of different experimental groups are shown in Table 1. Each dosimeter contained 3 ml TA with 1 µg/ml protoporphyrin IX and/or 0.030 mg/ml GNPs [48]. The groups receiving ultrasound were considered at single and dual frequency modes (0.8 and 2.4 MHz) with three exposure times and two different intensities. The experiments were also performed for groups under aforementioned conditions in the presence of scavengers of mannitol, histidine and sodium azide for 180 s sonication. All possible control groups were also considered.

Irradiation was performed using a flat transducer at continuous mode at 1 and 2 W/cm^2 intensities for three exposure times with one transducer in the presence and absence of three scavengers of mannitol, histidine and sodium azide.

The transducer was inserted under a perspex cylinder containing the dosimetric solution of TA. The cylinder containing TA solution was enveloped by a water jacket in the near-field of the ultrasonic probe at a distance of 5 mm from the surface of the probe [50, 51]. Figure 1 shows the ultrasound exposure set-up. The sonication times of TA solution were 20, 60 and 180 s. Under this setting, 2–3 °C increase in the temperature was observed. The measurements were performed on four TA solutions: TA solution with PpIX, TA solution with GNPs, TA solution with GNPs and PpIX, and TA solution with no additives. After ultrasound irradiation, the solutions were kept in a dark place and fluorometric assessments were performed 2–4 h after sonication. The fluorescence signal of solutions without ultrasound irradiation was subtracted from signals recorded from similar irradiated sample. Each experiment was repeated three times.

Table 1 The considered TA dosimeters in different experimental groups

Groups no	PpIX	GNP	US frequency (MHz)
1	–	–	–
2	+	–	
3	–	+	
4	+	+	
5	–	–	0.8
6	+	–	
7	–	+	
8	+	+	
9	–	–	2.4
10	+	–	
11	–	+	
12	+	+	
13	–	–	Dual frequency mode (0.8 and 2.4 MHz; 50:50)
14	+	–	
15	–	+	
16	+	+	

Fig. 1 Experimental set-up of ultrasound exposure

Statistical analysis

All data were analyzed using SPSS statistical software 13.0 (SPSS Inc., Chicago, IL, USA). The normality of data distribution was assessed by Kolmogorov–Smirnov normality test. Consequently, inter-group differences were compared using one-way ANOVA and Tukey tests at a significance level of 95%.

To determine the synergism index of dual frequency ultrasound, the recorded signals of dosimeters for dual ultrasound frequency were divided by the expected signal (sum of fluorescence signals related to similar samples irradiated by single frequency modes). Also, the change rate of fluorescence signals caused by scavengers was determined as below:

$$\text{Change in fluorescence signal} = \left(S - S_{sca}\right) \quad (1)$$

In the above equation, S and S_{sca} refer to recorded fluorescence signals in the presence and absence of scavengers, respectively.

Results

Figure 2 shows the fluorescence signal recorded in the TA solution contain PpIX, and/or GNPs, without any additives in the 0.8 MHz ultrasound. The data indicates a significant difference between 1 and 2 W/cm^2 ultrasound irradiations ($p < 0.001$). As can be seen, the fluorescence signal recorded from dosimeters at the frequency of 0.8 MHz with similar irradiation times is significantly higher at 2 W/cm^2 power density compared to 1 W/cm^2 ($p < 0.001$). The results of

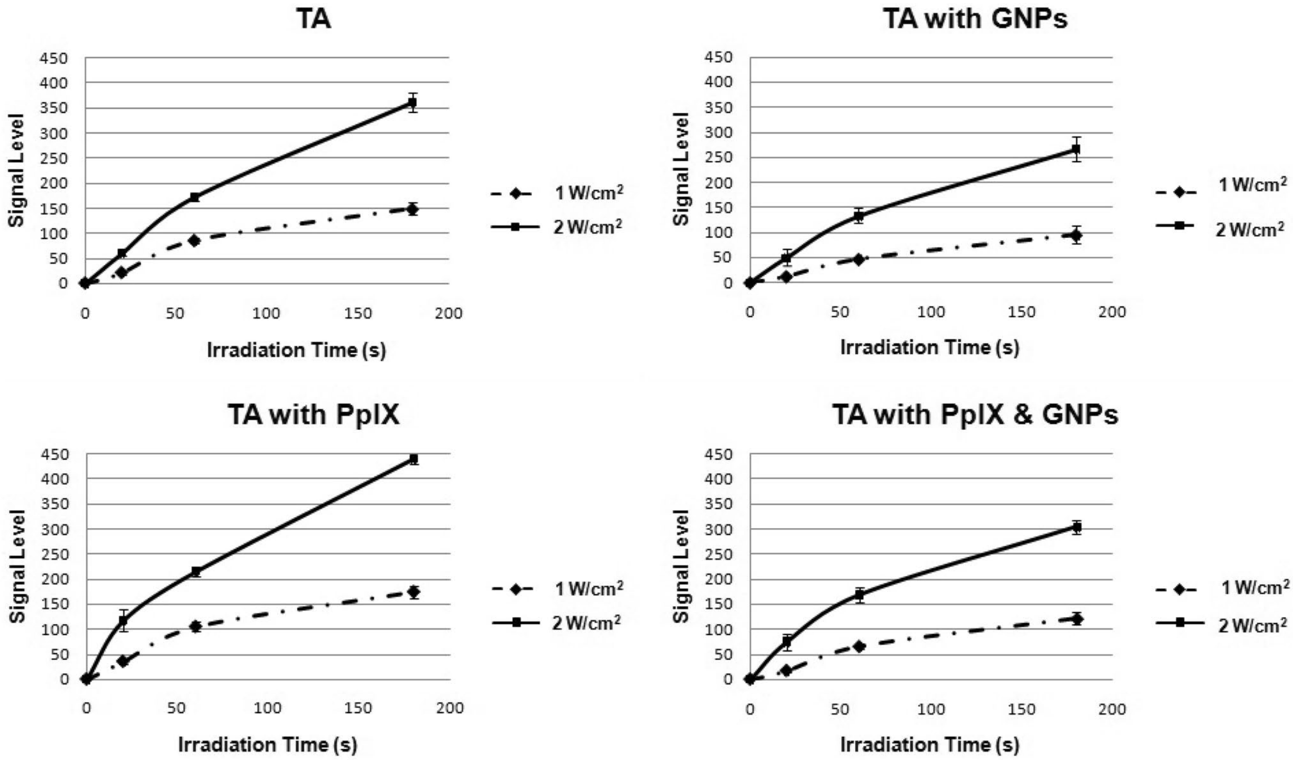

Fig. 2 Fluorescence signals generated after applying ultrasound with the frequency of 0.8 MHz frequency at 1 and 2 W/cm^2 power densities and different sonication times to TA dosimeters with and without GNPs and/or PpIX. The mean value of three experiments ± standard deviation is shown

statistical comparison show that an increase in the irradiation time is associated with increased fluorescence signal in all solutions ($p < 0.001$). Under similar conditions, compared to TA with no additives, which served as the group, the recorded fluorescence signals of dosimeters containing PpIX or PpIX with GNPs improved significantly, but in the presence of GNPs without PpIX, the dosimetric signal declined ($p < 0.001$).

Figure 3 shows the recorded fluorescence signal of dosimeters after irradiation by the ultrasound at the frequency of 2.4 MHz. Statistical analyses indicate the significant difference between two intensities of ultrasound at similar irradiation times ($p < 0.001$). The maximum and minimum signal levels were 180 and 20 s after radiation ($p < 0.02$). The fluorescence signal in the TA solution containing GNPs exposed to irradiation of 1 and 2 W/cm^2 ultrasound was significantly different in compared to other solutions without GNPs ($p < 0.001$). Also, at the 2.4 MHz at both intensities, the maximum fluorescence signal was recorded in the presence of GNPs that shows significant differences in comparison with and/or PpIX ($p < 0.004$). For the same ultrasound intensity and irradiation times, the addition of PpIX to TA solution did not show any significant differences on fluorescence signals compared to the TA solution without any additives ($p > 0.3$).

In Fig. 4, the fluorescence signals of dosimeters are shown for simultaneous irradiations by the dual frequency mode (0.8 and 2.4 MHz; 50:50). The level of fluorescence signal rose by increasing the exposure time and intensity of the TA solutions with and without additives ($p < 0.001$).

Table 2 shows the synergism index of dual frequency ultrasound. The synergism index greater than 1 shows additional cavitation activities during simultaneous application of two frequencies in comparison to application of a single frequency. The dual frequency ultrasound has intensified cavitation activity in the all samples.

The variations of fluorescence dosimetric signal for monitoring ROS generated by continuous ultrasound at different frequency modes were recorded for 180 s irradiation in the presence of three scavengers. Mannitol have long been studied as effective scavengers of $^{\cdot}OH$ radicals, NaN_3 is known as $^{\cdot}O_2$ scavenger and Histidine is an efficient scavenger of singlet oxygen and somewhat weaker scavenger of hydroxyl radicals. At the 2.4 MHz frequency, hydroxyl radicals are not generated during ultrasound irradiation in the absence of additive agents in TA or in the presence of PpIX. Accordingly, the variation of signal level with each scavenger is dependent on two factors of additive and frequency. At the 0.8 MHz frequency, the maximum reactive species are generated with PpIX. In the presence of GNP, the largest

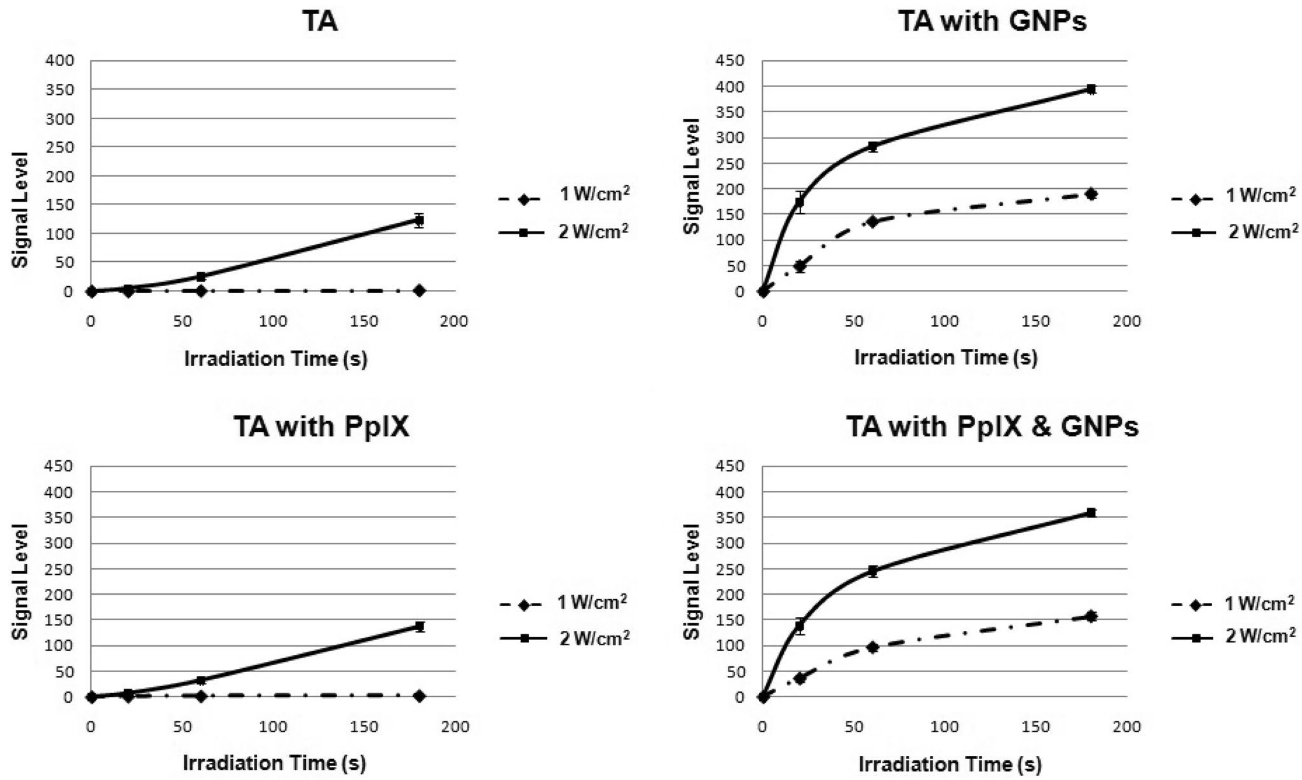

Fig. 3 Fluorescence signals generated after applying ultrasound with a frequency of 2.4 MHz at 1 and 2 W/cm^2 power densities and different sonication times to TA dosimeters with and without GNPs and/or PpIX. The mean value of three experiments ± standard deviation is shown

amount of $^{\cdot}OH$ and $^{\cdot}O_2$ are formed at the 2.4 MHz frequency followed by the dual frequency mode (Fig. 5).

Discussion

In this paper, the influence of different ultrasound modalities on cavitation activity was investigated to determine its effect on producing $^{\cdot}OH$ and $^1O_2$ in the presence of three scavengers, namely NaN_3, mannitol and histidine. Also, the cavitation potential of PpIX and/or GNPs was examined. Given the importance of GNP as a safe and effective carrier in drug delivery and targeting its exceptional acoustical, optical, and thermal properties, and also huge potential in combinational treatments of sonodynamic, photodynamic, and photothermal therapies further studies are required to clarify this point. Cavitation is known to be a non-thermal mechanism that accelerates chemical reactions [2, 38, 49]. To evaluate the impact of sonosensitizing agent and sonication parameters on cavitation activity, TA chemical dosimeters were utilized with GNPs and/or PpIX as additive agents. The generation of OH radicals by acoustic cavitation in an aquatic medium and their reaction with TA anion was monitored to produce fluorescent ions. HTA revealed an emission peak at a wavelength of 426 nm [40, 43]. We found that free radicals of OH and $^1O_2$ were significantly affected by divergent single and dual frequencies of ultrasound and type of additive agents.

The fluorescence signal of TA with and without adding PpIX at the 2.4 MHz frequency were weak and scavengers were unable to significantly change the signal level. At this frequency, our findings did not show any obvious OH^* production in TA alone. Therefore, 1 W/cm^2 ultrasound at the frequency of 2.4 MHz frequency with no additives in the dosimeter was below the threshold of cavitation and nanoparticles changed the situation.

PpIX increased the efficacy of OH radical generation by ultrasound irradiating at the 0.8 MHz frequency which was significantly higher than GNPs at the 2.4 MHz frequency. The production of hydroxyl radicals by PpIX at 0.8 MHz was higher than that found under other conditions. The minimum values were recorded at 2.4 MHz without additive and with PpIX. GNPs showed different behaviors at frequencies of 0.8 and 2.4 MHz. It reduced dosimetric signal at the frequency of 0.8 MHz, whereas the signal was reinforced at 2.4 MHz. In the study of Sazgarnia et al. [50], the fluorescence dosimetric signal was reinforced at the frequency of 1.1 MHz in the presence of GNPs (5–9 nm). In this study, the interaction of shorter ultrasound wavelengths with GNPs of 15 nm was more effective in generating and/or collapsing

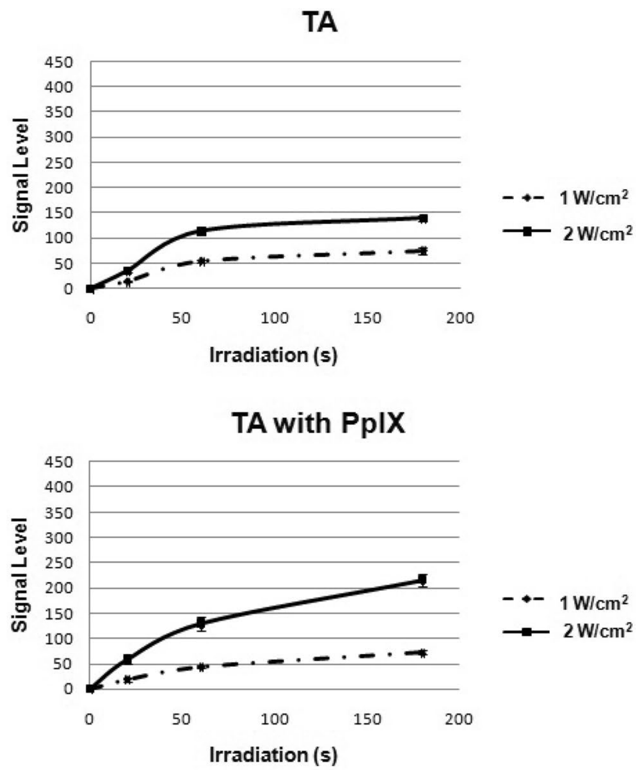

Fig. 4 Fluorescence signals generated after applying an ultrasound with a dual frequency mode (0.8 and 2.4 MHz; 50:50) at 1 and 2 W/cm^2 power densities and different exposure times to TA dosimeters with and without GNPs and/or PpIX. The mean value of three experiments ± standard deviation is shown

Table 2 Synergism index (SI) of dual frequency determined from the TA dosimeters signals with and without GNPs and/or PpIX

TA dosimeters with additive agents	No additive agent	GNPs	PpIX	PpIX & GNPs
Synergism Index (mean ± SD)	1.3 ± 0.2	1.7 ± 0.2	1.3 ± 0.1	1.6 ± 0.2

SI is a ratio of the signals after applying dual frequency ultrasound (0.8 and 2.4 MHz; 50:50) at 2 W/cm^2 power density to the sum of the signals obtained at the individual frequencies at 1 W/cm^2. Mean of SI has been calculated at different exposure times

bubbles. We expect that ultrasound frequency and GNP size are closely related to cavitation activity.

Increased fluorescence in the presence of PpIX-GNPs can be related to PpIX and GNPs, which acts as sonosensitizer and cavitation nuclei respectively. In other words, nanoparticles may serve as cavitation centers through which means cavitation activity is increased. Excited PpIX produces more free radicals. The significant difference between dosimeteric findings in the absence and presence of GNPs confirms the first assumption. This is in agreement with the study of Tuziuti et al. who showed particles in media provide the nucleation sites for cavitation bubbles due to their surface roughness, and decreases the cavitation threshold intensity and increases the quantity of bubbles during ultrasound exposure [52]. Our findings on PpIX was in agreement with the reports of Wang et al. and also Shanei et al. [38, 49]. Misik and Riesz showed that the sensitizer could be chemically activated and by the collapse of cavities, more energy was generated, and energy transfer from the activated sonosensitizer to oxygen molecule produced singlet oxygen [53]. Our findings showed that after ultrasound exposure, fluorescence signals of TA solutions in the presence of one of two agents—PpIX or GNP- was higher than in their absence. These results could be related to interaction mechanisms between ultrasound and the agents:

1. GNPs may have provided a central nucleus in support of cavitation that increased the rate of cavitation [37] or maybe GNPs created a rough surface that induced bubbles collapse and therefore enhanced the generation of free radicals. This effect was tangible at high frequency; whereas GNPs have suppressed cavitation at 0.8 MHz. Therefore, these findings can be due to local rise of temperature influenced by ultrasound absorption that resulted in the generation of the central vapor nucleus to

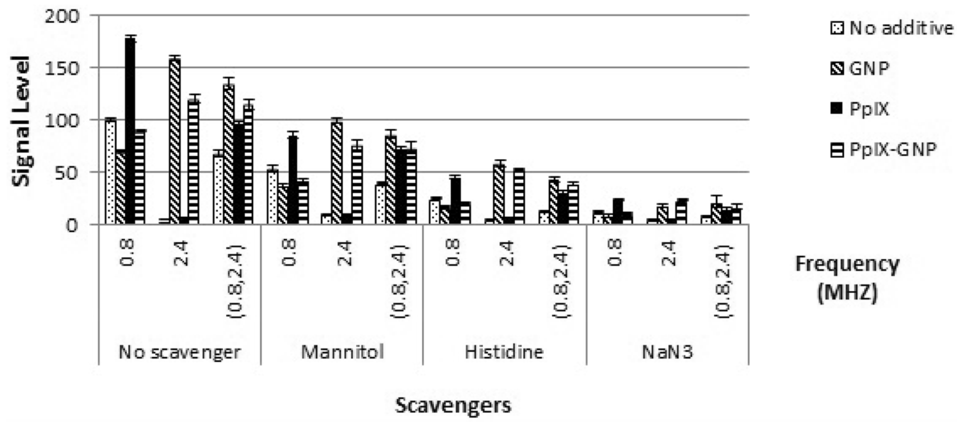

Fig. 5 The fluorescence signal recorded of TA dosimeters with and without PpIX or/and GNP for monitoring ROS generated by continuous ultrasound at different frequency modes after 180 s irradiation in the presence of three scavengers of mannitol, histidine or sodium azide

form cavitation. Ultrasound absorption and temperature increase in the vicinity of the GNPs at higher frequency is more than the low frequency.

2. More free radicals can be produced during the interaction of PpIX as a sonosensitizer with ultrasound with the chemical reaction of OH radicals enhancing fluorescence signal into the TA solution [54]. This effect was more noticeable at low frequency.

Finally, the dual frequency mode presented in this paper played the role of an effective producer of hydroxyl radicals in the presence of GNP, which was not more efficient than 0.8 MHz with PpIX or the frequency of 2.4 MHz with GNP. Also, dual frequency mode remarkably enhanced the fluorescence intensity of TA dosimeters, which was indicative of increased cavitation activity. This finding is in agreement with the report of Feng et al. [55] according to which the combination of two ultrasonic frequencies disturbed the solution more than the single-frequency irradiation, thus resulting in greater cavitation in the solution. In the study of Barati et al. the effect of exposure parameters on cavitation induced by low-level dual-frequency ultrasound was investigated [2]. They showed that simultaneous combined dual-frequency ultrasound in continuous mode was more effective in provoking cavitation activity at low-level intensity. This paper demonstrated the presence of a synergism utilizing dual frequency ultrasound with the activity of cavitation produced by simultaneous combined irradiation through two ultrasonic fields being higher than the summation of effects generated by either frequency individually. Therefore, dual frequency ultrasound can increase the effectiveness of sonodynamic processes. In vitro experiments on cancerous cell lines are needed to demonstrate the synergism in tissues. However, whether the use of harmonics is important or not is a question that should be investigated further. In this paper, we applied the first and third harmonics and the findings confirmed that cavitation activity intensified with increased intensity and sonication time and reduced frequency. These findings are consistent with several other reports [43, 49].

Conclusion

Regarding this research, PpIX was shown to be an effective sonosensitizer and 15 nm GNPs was shown to be an enhancer of cavitation activity at 2.4 MHz. PpIX was shown to be an effective sonosensitizer at 0.8 MHz, and dual frequency exposure was more effective at increasing the production of hydroxyl radicals. Two mechanisms are involved in the production of hydroxyl radicals in the presence of GNPs or PpIX.

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Compliance with ethical standards

Conflict of interest The authors report no conflict of interest in this research.

Ethical approval This article does not contain any studies with human participants performed by any of the authors.

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