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Beneficial Effects of Dual Frequency Sonodynamic Therapy and Hematoporphyrin Mesoporous Silica Nanoparticles in the Treatment of Mice Breast Adenocarcinoma

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ABSTRACT

Background: Sonodynamic therapy (SDT) may be a new hopeful non-invasive method for cancer treatment, which incorporates a combination of low-intensity ultrasound and a sonosensitive chemical. The goal of this study was to evaluate the effect of dual-frequency sonication (1 and 3 MHz) and injected Hematoporphyrin encapsulated in mesoporous silica nanoparticles (HP-MSNs) as a sensitizer in the treatment of mice grafted with breast adenocarcinoma.

Methods: In this research, one hundred and thirty-two female mice with grafted breast adenocarcinoma were separated into 22 groups including control, sham, 4 groups of sonication 1 or 3 MHz (1 and 2 W/cm^2), and 16 groups of SDT with Hematoporphyrin (HP) and HP-MSNs (2.5 and 5 mg/kg). The tumor growth factors and tumor grading were used to assess the treatment management.

Results: The results indicate that dual-frequency sonication has a delayed effect on tumor growth. The required time of T5 to the initial volume in all groups of SDT with HP (5 mg/kg) was greater than that in the control group ($P<0.05$). It was observed that SDT with an injection of HP-MSNs was effective in delaying tumor growth and the time of T2 and T5 was higher than that of other groups ($P<0.05$). This group had Grade II (intermediate), while others had Grade III (high) malignancy in the histological study of mice breast adenocarcinoma.

Conclusion: Our results reveal that dual-frequency SDT therapy with HP-MSN has a delaying tumor growth effect on mice breast adenocarcinoma. Hence, the expansion of minimally invasive methods such as SDT is necessary.

Keywords: Breast cancer, Adenocarcinoma, Dual frequency, Sonodynamic therapy, Hematoporphyrin, Mesoporous silica nanoparticle

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Introduction

After cardiovascular disease, cancer is a common cause of death within the United States and in many parts of the world (1). The most common cancer among women in Iran is breast cancer and among adolescent women is adenocarcinoma (2). It is often possible to treat breast cancer at an early stage, so considering the stage of cancer progression and therefore the subsequent selection of the acceptable treatment method is very important. Four main treatments commonly used for cancer are surgery, chemotherapy, radiotherapy, and immunotherapy. They have significant limitations and cost benefits; therefore, finding an efficient, safe and low-cost treatment is very necessary (3, 4).

Sonodynamic therapy (SDT) may be a new hopeful non-invasive method for cancer treatment, which incorporates a combination of low-intensity ultrasound and a sonosensitive chemical (3). Sonication, in addition to being non-hazardous, has the ability to penetrate deep into the tissue and may be concentrated in a small area of the tumor and effectively activate and increase cell sensitization and cytotoxicity (5). In SDT, ultrasound waves radiated to the tumor with an appropriate frequency and intensity. Therapeutic ultrasound (1-3MHz, 0.5-3 W/cm^2) has been employed due to tissue destruction with higher frequencies alongside cavitation. These waves interact with sonosensitizing chemicals and as a result, produce free radicals, which are toxic and cause apoptosis of cancer cells. In fact, this activation is related to the cavitation phenomena (6-8). This phenomenon involves the formation, growth, and exploding of gas-filled bubbles in fluids under appropriate conditions (3, 4). Due to the bursting bubbles, localized heat (about $1000^{o}k$) and high pressure (about 1000atm) are made over a brief period. There are sorts of cavitation, inertial cavitation, and non-inertial or stable cavitation (9, 10).

One of the most important SDT sensitizers which can maximize the ultrasound effects is Hematoporphyrin (4, 5, 11). Hematoporphyrin (HP) as an organic sensitizer, alone does not have any toxic effects (12). On the other hand, many sensitizers, including porphyrin-based molecules, are easily condensed into physiological environments due to their low solubility in water (13). A drug delivery system emerged to reduce existing constraints and increase therapeutic effects (14). Thanks to their small size (about 50-400 nm), nanoparticles can easily penetrate cell dams (such as membranes) and effectively accumulate the drug within the target tissue, thus minimizing damage to healthy tissues. Mesoporous silica nanoparticles are considered within the field of treatment and diagnosis (15). For the discharge of the drug-loaded into the mesoporous nano-carriers, the external stimulus of ultrasound is extremely considered because, in addition to activating sensitivities, allows the spatial and temporal control of drug release at the specified location; thereby, increases therapeutic benefits and reduces side effects (16-18).

Sonication with a combination of two or more frequencies has higher yields within the production of particularly unstable cavities, especially when the sonication frequencies are different. Additionally, the utilization of more than one frequency at low-intensity sonication increases the antitumor effects (19, 20). Many researchers revealed that sonication with dual-frequency ultrasound caused a greater number of active bubbles, which followed by cell death increase due to free radicals produced by a sonosensitizer drug (8, 10, 21). The goal of the present study was evaluating the effect of dual-frequency SDT (1 and 3MHz) and injected Hematoporphyrin encapsulated in mesoporous silica nanoparticles (HP-MSNs), as a sensitizer, in the treatment of Inbred Balb/C mice grafted with breast adenocarcinoma in terms of the parameters related to tumor growth, animal survival, and pathological examination of the tumor.

Materials and methods

Chemicals

The synthesis of MSNs was performed in the sol-gel process by application of an alkoxide precursor (tetraethyl orthosilicate: TEOS), and a surfactant (Cetyltrimethylammonium bromide: CTAB) (22). This method consists of the formation of mesoporous nanoparticles under the size range of 60-1000 nm (23, 24). The particles were dried at room temperature and calcined at $550^{\circ C}$ for 3h. Hematoporphyrin 50% (Sigma-Aldrich, Canada) was dissolved in PBS and stored in the darkroom at $4^{\circ C}$. Subsequently, Hematoporphyrin solution was placed adjacent to the synthesized nanoparticles. The Hematoporphyrin enters the mesoporous nanoparticle cavities in a passively process (25).

Tumor model

In order to use a syngeneic tumor model, the confirmed murine spontaneous breast adenocarcinoma was achieved from anesthetized Balb/C mice (ketamine/xylazine, 30 mg/kg, IP). The tumor tissue was chopped into fresh pieces with a diameter of 2-3 mm in PBS. A portion of tumor tissue was subcutaneously placed in the inguinal area of the receptor animal (Inbred Balb/C female mice, 6-8 weeks age) (26), and suture clips were used to close the incision. To prevent mice infection Cefazolin (200 mg/Kg) was dissolved in the animals' drinking water. All procedures performed in studies involving animals were in accordance with the ethical standards of the Research Ethics Committee of Semnan University of Medical Sciences (IR.SEMUMS.REC.1396.18).

Sonication

For sonication (S), the mice were anesthetized (ketamine/xylazine, IP) and then placed moveless by a specific holder in the near field of ultrasonic waves (30cm) in a cubic Plexiglas water tank ($25\times25\times35 cm^3$). Two ultrasonic probes (5 cm diameter) were fixed in a 90^o position and the central beam of each ultrasound wave was at right angles to the axis of the other. The first source was a 1 MHz (1, 2 W/cm^2) and the other source was a 3 MHz (1, 2 W/cm^2) ultrasonic treatment system (210P and 215A, Novin Medical Engineering, Isfahan, Iran). The time of the sonication process was 60 seconds.

Treatment groups

The treatment was started when each of the tumor diameters reached 7-10 mm. To evaluate the effect of dual-frequency ultrasound with injection of sensitizer on breast adenocarcinoma, one hundred and thirty-two female Balb/C mice were separated randomly into 22 groups (n=6) including control, sham (solvent injection), 4 groups of sonication: S 1 MHz (1 and 2 W/cm^2)+ 3 MHz (1 and 2 W/cm^2), 8 groups of SDT with Hematoporphyrin (HP): SDT 1 MHz (1 and 2 W/cm^2)+ 3 MHz (1 and 2 W/cm^2)+ HP (2.5 and 5 mg/kg), and 8 groups of SDT with Hematoporphyrin encapsulated in mesoporous silica nanoparticles (HP-MSNs): SDT 1 MHz (1 and 2 W/cm^2)+ 3 MHz (1 and 2 W/cm^2)+ HP-MSN (2.5 and 5 mg/kg). Due to the weight of experimental animals (20 ± 2 g), a dose of 10 mg/kg (0.2 ml) HP, or HP-MSNs was injected intra-peritoneally 24h before sonication (26).

Evaluation of the anti-tumor effect

After SDT performance, to evaluate the tumor volume, the length (a), width (b), and depth (c) of each tumor was measured with a digital caliper every 3 days and mass volume was estimated from the volume formula: $V= 0.5 \times a \times b \times c$. The calculated volumes (V) were used to evaluate tumor growth parameters: relative volume (Relative volume = $[(V-V_o)/V_o] \times 100$), tumor growth inhibition ratio (TGI %= $[1- (V_{x\ day} /V_{cotrol\ day})] \times 100$), and the times needed for each tumor to reach two (T2) and five times (T5) to the primary mass volume (20).

Histopathological images of mass sections were obtained over 30 post-treatment days. Tumor sections were stained with hematoxylin/eosin to assess tumor grading and malignancy based on Bloom-Richardson (BR) classification (tumor tubule formation, the number of mitosis/10 high power fields, and nuclear grade). The degree of tumor grading was low grade (well-differentiated), intermediated grade (moderately-differentiated), and high grade (poorly-differentiated) (27). The histopathological analysis was performed blindly.

Statistical analysis

Statistical analysis was performed through SPSS 16.0 software, normality distribution using the Tukey test, and the statistical differences by one-way ANOVA ($P < 0.05$). The data were expressed as means ± SD.

Results

Results obtained from the relative tumor volume after treatment with dual-frequency sonication have been plotted in Figure 1. These results indicate that sonication has a delayed effective tumor growth in comparison with the control and sham groups after 15 days of treatment. Analysis of data showed non-significant differences between groups prior to 15 days ($P>0.05$). Comparison of data showed non-significant differences between groups to reach two-times and five-times the initial volume in the presence of sonication ($P>0.05$). The tumor growth inhibition percent (TGI %) over 30 days of treatment is shown in Figure 2. Inhibition of tumor growth in the groups treated with sonication was greater than that of the sham. The tumor growth inhibition ratio increased by 39-48% in sonication groups after 9 days of the initiation of the treatment. The experiment demonstrated that this increase was transient and declined over 30 days of treatment.

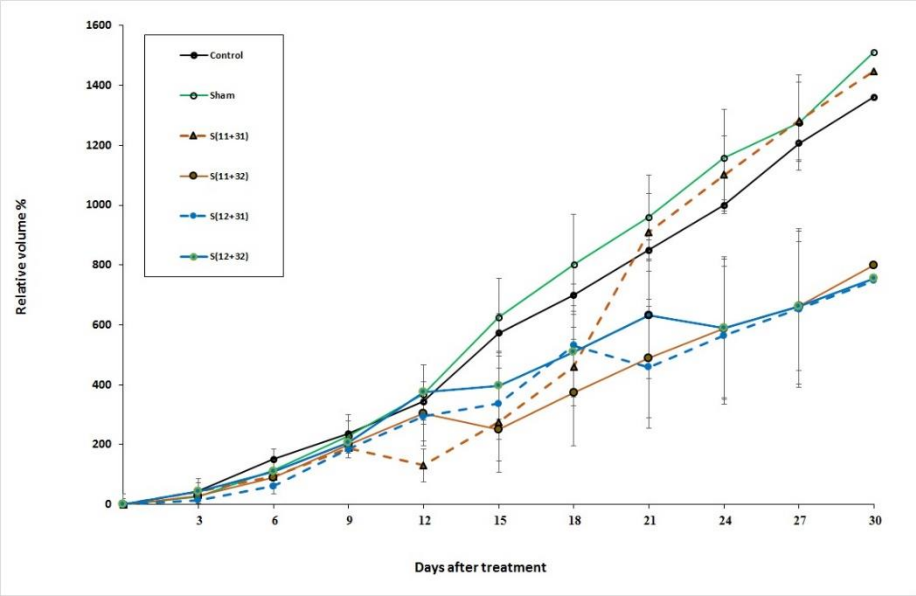

Figure 1. The mean ± SD of the relative volume percent of adenocarcinoma tumors for control, sham, and dual-frequency (1 and 3 MHz) sonication groups

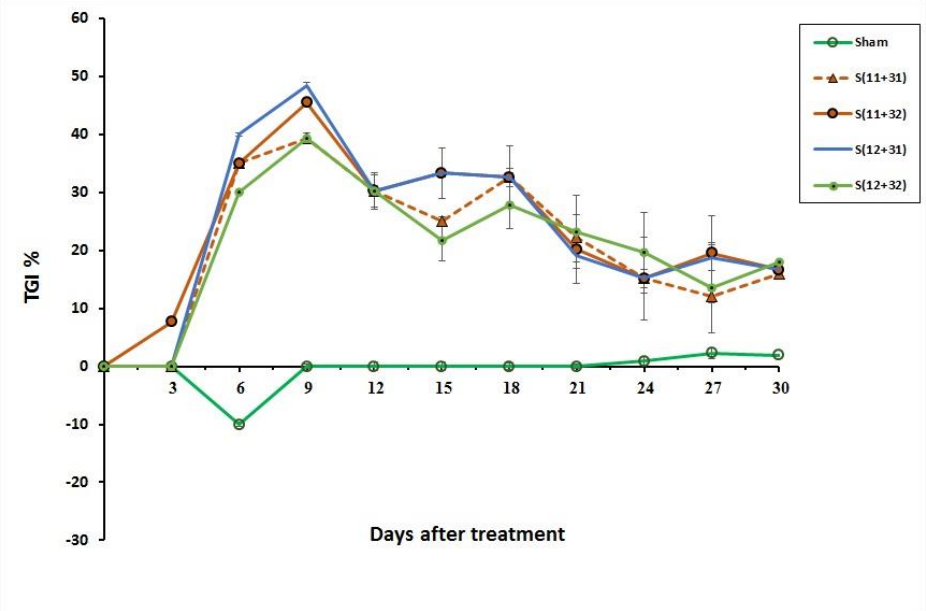

Figure 2. The tumor growth inhibition percent (TGI %) in the following treatment groups: sham and dual-frequency (1 and 3 MHz) sonication groups

To validate our findings, we estimated the effects of SDT with HP (2.5 and 5 mg/kg). Figure 3, demonstrates the post-treatment relative tumor volume. Significant differences were observed between experimental groups and sham in tumor volume, 15 days after the treatment ($P<0.05$). The minimum relative tumor volume percent belonged to SDT with HP (5 mg/kg) groups. Analysis of T2 data showed non-significant differences between groups ($P>0.05$). The required time of T5 to the initial volume in all groups of SDT with HP (5 mg/kg) was greater than that of the sham group ($P<0.05$). As presented in Figure 4, the tumor growth inhibition percents of these groups on the 18th post- treatment day were 32-48%. Figure 5, shows tumor growth curves based on the relative volume percent during the 30 post-treatment days. These results indicate that SDT with an injection of HP-MSNs is effective in delaying tumor growth when compared with the sham group ($P<0.05$). The time of T2 in the SDT 1 MHz (2 W/cm^2) + 3 MHz (2 W/cm^2) + HP-MSN (5 mg/kg) group was greater than that in other

groups (P<0.05). In addition, the required time of T5 to the initial volume in all groups was greater than that in the sham group (P<0.05). As the T2 findings, T5 in the SDT 1 MHz (2 W/cm^2) + 3 MHz (2 W/cm^2) +HP-MSN (5 mg/kg) group was larger than that in other groups (P<0.05).

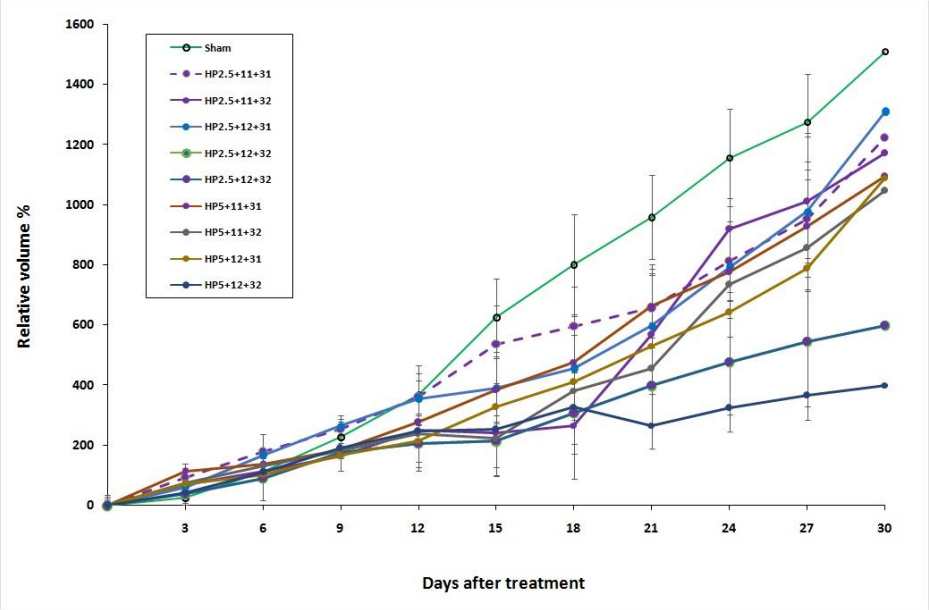

Figure 3. The mean ± SD of the relative volume percent of adenocarcinoma tumors for the following treatment groups: sham and dual-frequency (1 and 3 MHz) SDT with injection of HP (2.5 and 5 mg/kg) groups

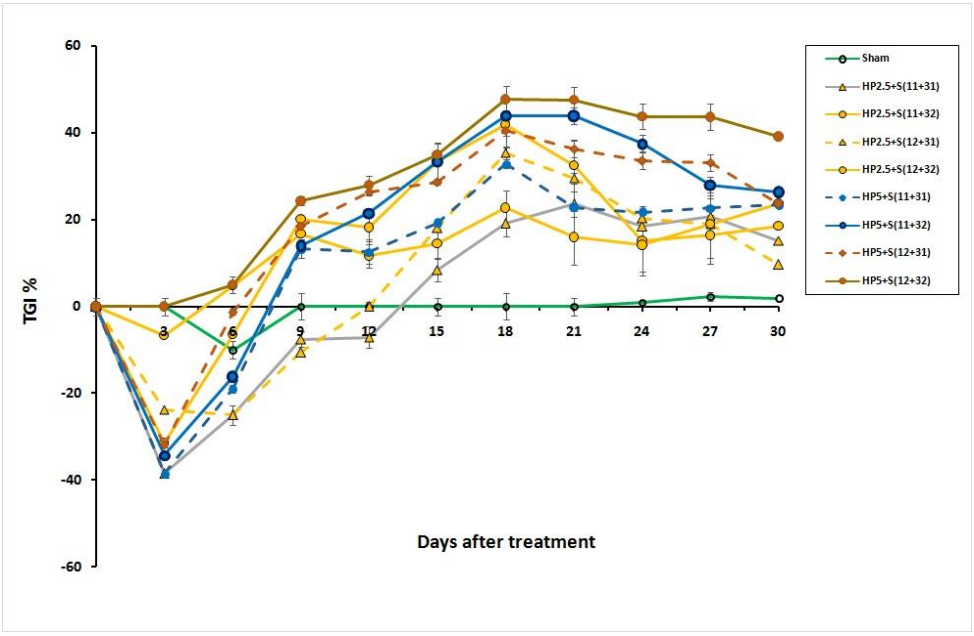

Figure 4. The tumor growth inhibition percent (TGI %) in the following treatment groups: sham and dual-frequency (1 and 3 MHz) SDT with injection of HP (2.5 and 5 mg/kg) groups

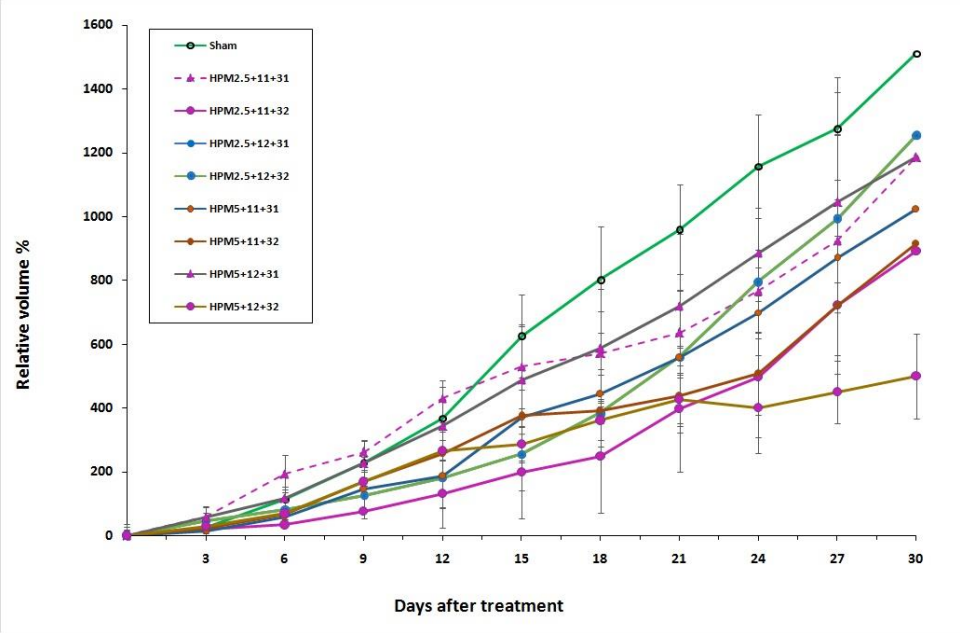

Figure 5. The mean ± SD of the relative volume percent of adenocarcinoma tumors for the following treatment groups: sham and dual-frequency (1 and 3 MHz) SDT with injection of HP-MSNs (2.5 and 5 mg/kg) groups

Figure 6, demonstrates tumor growth curves during 30-day post-treatment period. Inhibition of tumor growth (TGI %) in the groups treated with SDT and injection of HP-MSN was higher than that in sham group ($P<0.05$). The tumor growth inhibition ratio increased in all groups on the 6th day after the initiation of the treatment. The TGI% of all groups with SDT and HP-MSNs (5 mg/kg) on the 18th day after the treatment was 32-44%. As shown in Figure 7, Kaplan-Meier analysis presented experimental groups' survival in terms of the cumulative survival function. The maximum survival probability (95%) for the group treated with SDT (2, 2 W/cm^2) and HP-MSN (5 mg/kg) was 51 days; while, for control, SDT (2, 2 W/cm^2), HP-MSN (5 mg/kg), and SDT 1 MHz (2 W/cm^2)+ 3 MHz (2 W/cm^2)+HP (5 mg/kg) were 31, 34, 39, and 41 days respectively.

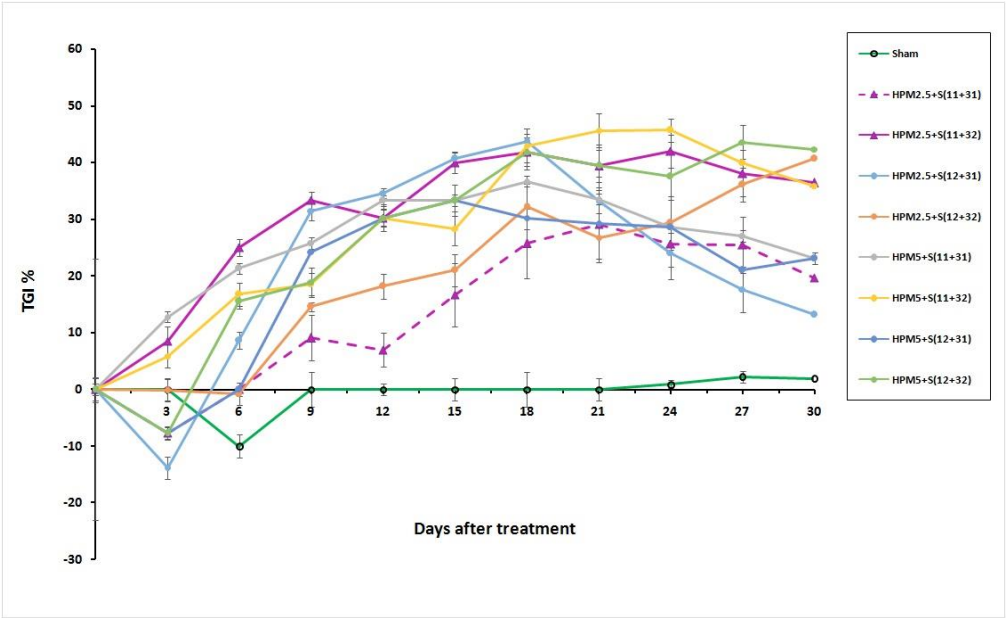

Figure 6. The tumor growth inhibition percent (TGI %) in the following treatment groups: sham and dual-frequency (1 and 3 MHz) SDT with injection of HP-MSNs (2.5 and 5 mg/kg) groups

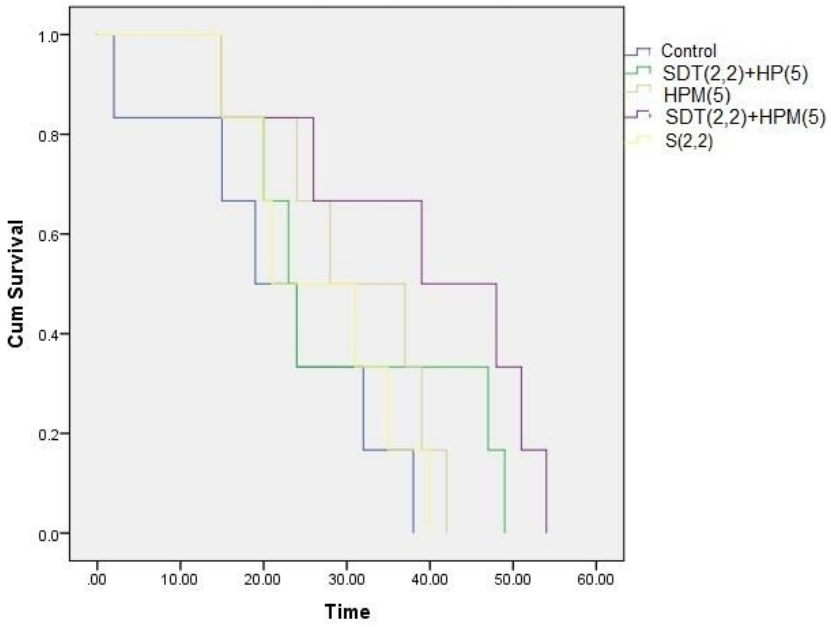

Figure 7. Kaplan-Meier curve comparing the cumulative survival function (days) between control, sonication (2, 2 W/cm^2), HP-MSN (5 mg/kg), SDT (2, 2 W/cm^2) + HP (5 mg/kg), and SDT (2, 2 W/cm^2) + HP-MSN (5 mg/kg) experimental groups, 50 days after treatment

Microscopic assessment of tumor sections revealed multiple nuclear mitosis and polymorphism in all investigational groups (Figure 8). The results of the histopathological study to determine the grading of the tumors were shown in Table 1. All experimental groups had Grade III (high) malignancy, apart from SDT 1 MHz (2 W/cm^2) + 3 MHz (2 W/cm^2) + HP-MSN (5 mg/kg) group which had Grade II (intermediate) in the histological study of mice breast adenocarcinoma.

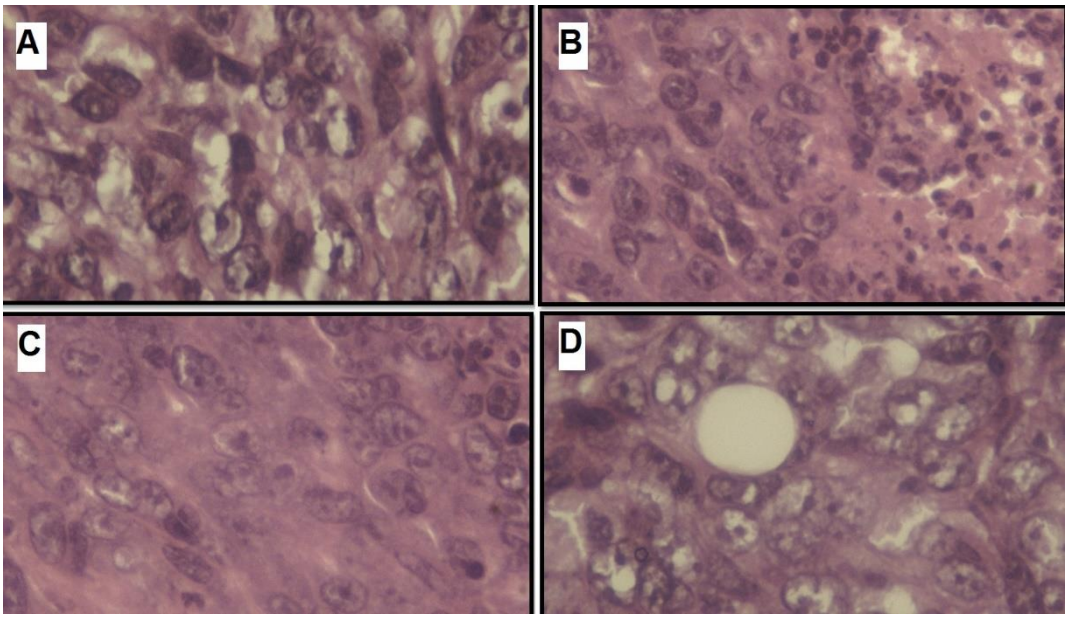

Figure 8. Histopathological images of tumor tissue sections, A: control, B: HP-MSN (5 mg/kg), C: sonication (2, 2 W/cm^2), and D: SDT (2, 2 W/cm^2) + HP-MSN (5 mg/kg) experimental groups.

Table 1. Bloom-Richardson (BR) classification of tumors in the control, sham, HP-MSN (5 mg/kg), dual-frequency sonication (2, 2 W/cm^2), and dual-frequency SDT (2, 2 W/cm^2) + HP-MSN (5 mg/kg) experimental groups.

Group	Tumor tubule formation	Number of mitosis/10 high power field	Nuclear grade	Total score	BR grade	Grade
Control	3	3	3	9	Poorly Differentiated	3
Sham	3	3	3	9	Poorly Differentiated	3
HP-MSN (5 mg/kg)	2	3	3	8	Poorly Differentiated	3
Dual-frequency Son (2 ,2 W/cm^2)	3	2	3	8	Poorly Differentiated	3
Dual-frequency SDT (2,2 W/cm^2) + HP-MSN (5 mg/kg)	2	2	2	6	Moderately Differentiated	2

Discussion

In this research, the effect of dual-frequency SDT (1 and 3 MHz) and injected HP-MSN (2.5 and 5 mg/kg) as a sensitizer in the treatment of Inbred Balb/C mice breast adenocarcinoma was evaluated. The results indicate that dual-frequency sonication (1 + 3 MHz) has a delayed effect on tumor growth. This finding is in agreement with Barati *et al.* findings that dual sonication (1 MHz + 150 kHz) for 30 min decreased mice breast adenocarcinoma tumor growth (28). In addition, Guan and Gang showed that high-intensity focused ultrasound (1.6 MHz) could destroy proliferating tumor cells in human breast cancer (29). In a study, anti-tumor effect of SDT with HP (2.5 and 5 mg/kg) in the treatment of mice breast adenocarcinoma was assessed and the minimum relative tumor volume percent belonged to the SDT + injection of HP (5 mg/kg) group. In accord, evaluation of the combination of dual-frequency ultrasound (1 MHz + 150 kHz) and HP (5 mg/kg) resulted in a significant reduction in the relative volume percent of mice breast adenocarcinoma (20). Several studies have been administered on the consequences of synergistic ultrasound and sensitizers. The results of the Umemora study indicated that ultrasound radiation with the injection of HP caused tumor growth inhibition (12). Moreover, Yue demonstrate that SDT (1 MHz) with HP monomethyl ether eliminated the 4T1 murine breast cancer cell line (30). Quan-honget and Lv concluded that use of SDT with HP, resulted in at least three times higher toxicity than that of ultrasound and HP alone (31, 32).

In the present study, to estimate the effect of dual-frequency SDT with HP-MSN on breast adenocarcinoma, the relative volume of the mass was evaluated during 30 post-treatment days. The results indicated that SDT with an injection of HP-MSN (5 mg/kg) is effective in delaying tumor growth. These data are the same as the results of our previous investigation with SDT 3 MHz (33); that is, the results of SDT are not frequency-dependent and not only are determined by sonication wave power density, but also are related to HP-MSN injection dose. In both frequencies, the tumor malignancy declined with an increase in sonication power density and HP-MSN injection dose. In agreement with our results, Zheng concluded that the therapeutic effect of encapsulated HP-SDT was better than that of HP or ultrasound alone (16). In addition, during a study by Hasanzadeh, to review the effect of dual-frequency ultrasound radiation on nanomicellar containing doxorubicin, the combination of ultrasound radiation increased the cavitation efficiency (8). Although in our findings SDT with HP and HP-MSNs had an inhibition effect on tumor growth, the histopathological results (Bloom-Richardson classification basis) showed that all experimental groups had Grade III (high) malignancy, apart from SDT 1 MHz (2 W/cm2) + 3 MHz (2 W/cm2) + HP-MSN (5 mg/kg) group which has Grade II (intermediate) in mice breast adenocarcinoma. This might be due to the excellent features of mesoporous silica as high surface area, high pore volume, and great loading capacity of drug delivery (14).

According to the results of a study by Yumita, HP has the sensitizing effect and does not have a toxic effect within the absence of an external stimulus (11). One of the limitations of porphyrin-based sensitizers is their limited solubility in water and consequently their accumulation in the physiological environment. Nanoparticles utilized in drug delivery systems

have important advantages like increasing the buildup of drug molecules within the patient's tissues and cells (14). Many studies have been performed to evaluate MSN as a drug carrier. In an in vitro and in vivo study, no immunological sensitivity was reported for these nanoparticles, and MSNs were shown to possess low apoptotic cytotoxicity and cell death. These nanoparticles have great potential for biomedical applications and biotechnology due to their good biology (34).

It has been mentioned that sonication is a non-invasive technique with non-ionizing radiation which is cost-effective, and has an easy tissue penetration effect (14). However, Therapeutic ultrasound (1-3MHz, 0.5-3 W/cm^2) has been employed due to tissue destruction with higher frequencies (7). Acoustic cavitation is the main cause of destructive chemical reactions and free radical production since ultrasound irradiation. During a study by Feng, the effect of mixing two-frequency and three-frequency ultrasonic waves on cavitation efficiency was investigated. The results showed that irradiation of two or more ultrasound waves significantly increases the cavitation efficiency compared to single-frequency irradiation (19). Also, the investigation demonstrates that dual-frequency sonication could have a greater number of active bubbles compared to a single-frequency ultrasound (21, 35).

The synergy of sensitizer and sonication comprises several mechanical, chemical, and cavitational activated mechanisms (28). The structure of mesoporous channels would reveal drug release by sonication through a mechanical process and increases the cavitation effect (36). Sonication would enhance the sensitizer action by increasing the cell membrane's permeability and the capacity of sonication to improve solid tumor chemotherapy (37). Hence, the interaction of nanoparticles with ultrasound waves can produce acoustic cavitation. The collapse of cavitation bubbles can cause sono-mechanical and sono-chemical cytotoxic effects, as the formation of cytotoxic reactive oxygen species (38), including singlet oxygen and hydroxyl radicals (the further formation of H_2O_2 and peroxyl radicals), could kill tumor cells via apoptosis and necrosis (39). This means that the combination of SDT and HP-MSNs could have a better treatment effect on mice breast adenocarcinoma. Moreover, advanced investigations and future experiments should be accomplished to find better tumor treatment methods and explain the mechanism of these occurrences.

Conclusion

In conclusion, the findings of this study demonstrated that dual-frequency SDT 1 and 3 MHz with an injection of HP-MSNs (5 mg/kg) have an anti-tumor effect in mice breast adenocarcinoma. It can be appreciated that careful selection of the nanoparticle with sonication will play a useful function in the success of minimally invasive cancer therapies.

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