

Sonodynamic therapy of breast cancer cells using 1 MHz ultrasound: impact of gold nanoparticles

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ABSTRACT

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Background: Sonodynamic therapy (SDT) has emerged as a promising adjunctive treatment in cancer therapy, leveraging ultrasound and sensitizers to generate reactive oxygen species and induce tumor cell death. This study evaluated the efficacy of SDT with and without gold nanoparticles (NPs) as sensitizers on two breast cancer cell lines. **Materials and Methods:** Using gold NPs, MCF7 and MDA-MB-231 breast cancer cells were incubated and subjected to ultrasound irradiation at 1 MHz. In the case of treatment, ultrasound alone and ultrasound combined with NPs were taken into consideration. Cell viability was measured 48 hours post-treatment using the MTT assay. **Results:** There were significant differences in cell viability between the treated and control groups. For MCF7 cells, a 1-minute continuous ultrasound reduced survival to 60% ($p < 0.05$), and MDA-MB-231 cells showed 65% viability under the same conditions ($p < 0.05$). Therefore, enhanced therapeutic effects were detected by the use of gold NPs. **Conclusion:** SDT, combined with gold NPs, reduced breast cancer cell viability, particularly. This approach could potentially offer a safer alternative to conventional therapies by minimizing side effects while maintaining therapeutic efficacy.

INTRODUCTION

Despite advancements in diagnosis and treatment, cancer remains a major global health challenge. Breast cancer has a high prevalence, malignancy, and recurrence ⁽¹⁾. Surgery, chemotherapy, and radiation are common treatments, but drug resistance and recurrence are risks. Surgery removes tumors, radiation damages healthy cells, and chemotherapy affects healthy cells ⁽²⁾. While ultrasound is widely used in diagnostics, its therapeutic applications in cancer treatment remain underexplored ⁽³⁾. Cancer cells developing resistance to treatments pose challenges ⁽⁴⁾. New therapies are crucial for improving breast cancer outcomes, especially for aggressive types like triple-negative breast cancer. Sonodynamic Therapy (SDT) was introduced to the medical community in 1995 as a method using ultrasound waves with sound-sensitizing drugs to produce reactive oxygen species and cause cell damage ⁽⁵⁾. Sound sensitizers are essential for high ultrasound beam absorption in cells with low toxicity and high targeting ⁽⁶⁾. SDT utilizes cavitation and sonochemical reactions to treat cancers by causing biological damage through free radicals. It is known for the synergistic effect of sound sensitizers and ultrasound waves, relying on non-thermal effects like unstable cavitation ⁽⁷⁾. The main components of SDT are ultrasound waves and a sound sensitizer, activating a chemical sensitizer through bubble

collapse to produce free radicals ⁽⁸⁾. Metal NPs, especially gold NPs, have been proposed as sound sensitizers in SDT due to their non-invasive and painless treatment method for various diseases ^(9,10). The synthesis and evaluation of various gold NPs have gained interest from scientists ⁽¹¹⁾. Current studies highlight the advantages of NPs due to optimized protocols producing different sizes and shapes with unique properties. NPs can be modified with targeted compounds for diverse biomedical applications, especially in cancer treatment ⁽¹²⁾. Gold NPs offer controllable distribution patterns and good compatibility, making them promising for new treatments ⁽¹¹⁾. Acoustic sensitizers (NPs) in Sonodynamic Therapy based on SDT produce reactive oxygen species (ROS) through cavitation and thermal decomposition. Unstable cavitation leads to ROS production during the collapse of bubbles in the Pyrolysis method. ROS generated reacts with water molecules in the cell cytoplasm, contributing to apoptosis through oxidative stress ⁽¹³⁾. Effective cancer treatment strategies involve inhibiting growth and inducing apoptosis in cancer cells ⁽¹⁴⁾. Breast cancer treatment side effects from chemotherapy and radiation therapy are unpleasant. Solid tumor hallmark hypoxia leads to resistance against oxygen-dependent treatments. Despite advancements in breast cancer treatment, recurrence rates are high due to resistance mechanisms like radiotherapy ⁽¹²⁾. Therefore, it is necessary to develop a new strategy to

treat these cancers. The synergistic effect of cytotoxicity of the two physical modalities of ultrasound and X-megavoltage and its exact mechanism in breast cancer remains unclear and limited^(15, 16).

In the current investigation, the utilization of gold NPs is explored as a viable acoustic sensitizer in the context of ultrasound waves to diminish the survival rate of breast cancer cells. The novelty of the work is the combination of sonodynamic therapy with gold NPs using 1 MHz ultrasound to enhance selective breast cancer cell destruction.

MATERIALS AND METHODS

Cell culture

In this study, informed consent was obtained for the examination, which was either commercially available or provided by a recognized biorepository, and the ethical committee of Tarbiat Modares University approved the protocol of the study (IR.MODARES.REC.1400.015). MCF7 and MDA-MB-231 breast cancer cell lines were acquired from the National Cell Bank of Iran (NCBI, Pasteur Institute of Iran) and cultured in 25 cm² flasks. The cells were grown in Dulbecco's Modified Eagle Medium (DMEM-Sigma-Aldrich) with 4.5 g/l glucose, supplemented with 10% (v/v) fetal bovine serum (FBS-Sigma-Aldrich), 2 mmol/L L-glutamine, and antibiotics (100 µg/ml penicillin, 100 µg/ml streptomycin), under humidified air with 5% CO₂ at 37 °C. The cytotoxic activity was evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich). Dimethyl sulfoxide (DMSO-Sigma-Aldrich) and phosphate-buffered saline (PBS-Sigma-Aldrich) were obtained from Fisher Scientific.

Synthesis of gold NPs

In the process of synthesizing gold NPs, Turkevich's regeneration technique was employed as outlined in the literature reference⁽¹⁷⁾. This method involves the utilization of sodium citrate for regeneration. To initiate the synthesis of gold NPs utilizing this established methodology to produce a suspension of 100 ml of gold NPs, the initial step involved the meticulous preparation of a fresh 0.01 percent solution of tetrachlorauric acid (HAuCl₄·3H₂O) sourced from Sigma, a renowned supplier based in Germany, in 4-fold distilled water. Furthermore, a single milliliter of trisodium citrate solution (Na₃C₆H₅O₇) procured from Merck in Germany, with a concentration of 1%, was meticulously prepared as a reducing agent and freshly made for immediate use. The gold solution was subjected to a controlled heating process within an appropriate vessel. At this juncture, the solution underwent simultaneous agitation using a suitable high-speed bar magnet until it achieved the boiling point. Subsequently, the reducing solution was

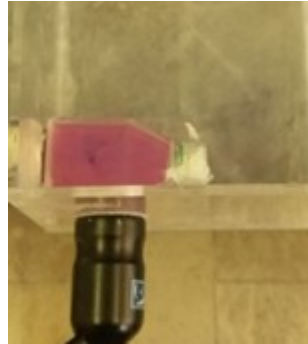
meticulously introduced into the solution in a gradual, dropwise manner, following which the resultant solution was allowed to gradually cool in an unobstructed atmospheric environment. After these steps, a 0.05 percent by-weight solution of polyvinyl alcohol, sourced from Merck in Germany, possessing a molecular weight of 3 kilodaltons, was introduced into the stirring solution to facilitate stabilization. To yield gold NPs with a desired diameter below 50 nm, the volume of reductant utilized was determined based on insights gleaned from prior experimental studies. The evaluation of the size and morphological characteristics of the synthesized gold NPs was accomplished through the utilization of a scanning electron microscope apparatus featuring a tube voltage of 100 kV, manufactured in Germany. Additionally, the analysis of the size distribution of the NPs was meticulously conducted with the assistance of specialized Image Tool software. In this study, four groups of experiments were performed: sonication with 1 MHz (1 W/cm²), the AuNPs, sonication with 1 MHz, and the AuNPs, control. Each treatment group was repeated three times. The cell groups were sonicated with a frequency of 1 MHz at 1 W/cm² for 30, 60, 120, 150, 300, 600, and 1200 seconds. Therapeutic ultrasound irradiation of the cells was performed using the SM-3670 model therapeutic ultrasound device manufactured by Shrewsbury Medical Co., based in Shrewsbury, UK. This device can provide a maximum continuous mode intensity of 1 W/cm² and a pulsed mode intensity of up to 2 W/cm², with selectable duty cycles of 0%, 25%, 33%, 50%, and 100%. Note that the temperature was measured during ultrasonic irradiation, using a micro thermocouple (±1 °C).

Cell culture flask temperature monitoring

Because the utilization of ultrasound waves, particularly those of high frequency, for therapeutic purposes is accompanied by the phenomenon of heat generation, it becomes imperative to delve into the intricacies of how the temperature escalates during exposure to ultrasonic waves within a cell flask. This necessitates the implementation of a meticulous temperature monitoring procedure. The current research endeavor undertakes the task of gauging the temperature within a cellular flask containing water, positioned within one of the thermometer channels, ensconced within a perspex water phantom. The apparatus employed for temperature measurement is a sophisticated 2-channel digital thermometer, whereby one channel interfaces with the flask while the other interfaces with the phantom environment. These thermocouples establish a crucial linkage with the thermometer device, thereby facilitating the acquisition of temperature data. Before commencing the experimental procedures, due diligence is exercised to ensure the accuracy of the thermometer readings. This is achieved through a meticulous

calibration process involving the immersion of the thermometer in a solution comprising water and ice, set at a reference temperature of zero degrees. The spatial configuration of the probe, as well as the methodology employed for situating the thermometer within the flask and subsequently placing the flask within the water phantom, adheres meticulously to the guidelines delineated in figure 1 of the experimental setup.

Figure 1. The experimental setup.



Throughout the course of the experimentation, a mercury thermometer is concurrently utilized as an additional measure of assurance, serving the purpose of corroborating the temperature readings obtained from the digital thermometer.

Assessment of acoustic sensitization and reproductive toxicity of gold NPs

The enhancement of ultrasound treatment efficacy with the presence of pegged gold NPs and the evaluation of the potential toxicity of these NPs on MCF-7 and MDA-MB-231 cells were conducted through the utilization of the MTT (2,5-diphenyltetrazolium bromide) assay, a common method for measuring cellular viability. The Tetrazolium Bromide assay serves as a colorimetric indicator that assesses the overall health of cells, reflecting their growth and division capabilities. The MTT compound initially appears yellow and undergoes a transformation into a purple formazan derivative within the mitochondria of viable cells. This conversion process is reliant on the enzymatic activity of reductase enzymes, specifically those involved in the respiratory cycle within the mitochondria. Hence, the extent of purple formazan generation correlates directly with the number of viable cells present. By comparing the quantity of formazan produced by treated cells to that of untreated cells (control group), the impact of the treatment agent on cell viability and potential induction of cell death can be determined. To execute this experimental procedure, following a 48-hour irradiation period, 20 microliters of MTT solution with a concentration of 5 mg/ml were introduced into each well of a 96-well plate containing the irradiated cells alongside the control cell samples. Subsequently, the plates were incubated for a duration of 4 hours within an incubator set at a

constant temperature of 37 °C and 5% CO₂. Upon completion of the incubation period, the culture medium containing MTT was aspirated from the wells, and 100 microliters of DMSO (dimethyl sulfoxide) were dispensed into each well. The quantification of light absorption was then performed using an ELISA reader (Vira Elisa Microplate Reader, Iran), measuring the optical density at a wavelength of 570 nm to assess the formazan levels generated within the cells under investigation.

Statistical Analysis

Data were expressed as mean $\pm$ standard deviation (SD). One-way ANOVA followed by a t-test was used to assess statistical significance. Noted that a P-value of less than 0.05 was considered statistically significant. Statistical analyses were performed using SPSS version 26.0.

RESULTS

The size and appearance of gold NPs

Based on the scanning electron microscope (SEM) images obtained for the samples under investigation, it was clearly demonstrated that the NPs produced exhibit a nearly spherical morphology. Furthermore, thorough examination and evaluation of the images utilizing advanced image analysis software, namely ImageJ, revealed that the mean diameter of the particles measures approximately 14 nanometers (with the size estimation procedure being repeated a total of 50 times to ensure accuracy and precision). Additional comprehensive information and insights regarding the findings can be found in figure 2.

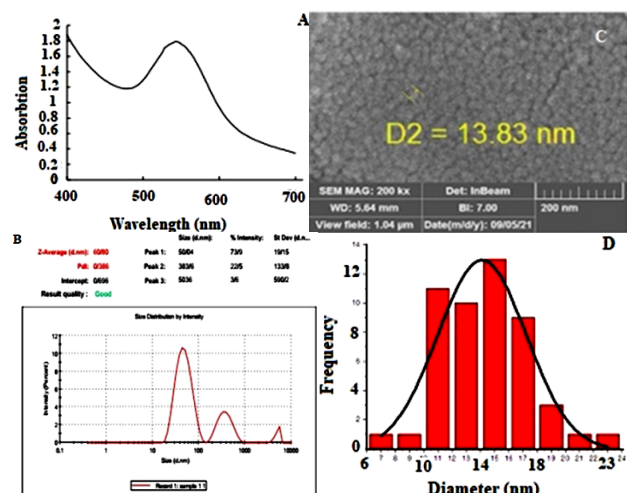


Figure 2. (A) the absorption spectrum of the synthesized particles; (B) the DLS result of the synthesized particles; (C) is a scanning microscope scan, and (D) is the size distribution of the synthesized particles (approximately 50 times sampling).

Figure 3 shows the curve related to the temperature changes inside the flask of the cell, which was located at a distance of 1 cm from the generator and was filled with water around and

inside. Each row shows the temperature response at the specified intensity levels under the corresponding mode of sonication. Analysis of the aforementioned graphs leads to the deduction that the duration required to achieve 1 °C temperature variance within the flask, in order to mitigate thermal impacts and adhere to the hyperthermia threshold for 1 MHz radiation in this particular investigation, is 60 seconds and intensity 1 W/cm² in continuous mode. The obtained results from temperature monitoring diagrams demonstrated that the mode and intensity of ultrasound exposure significantly influenced thermal effects within the cell flask. At low intensity (0.1 W/cm²), both continuous and 40% pulsed modes show minimal temperature rise, indicating negligible thermal impact. However, at higher intensities, 1 W/cm² in continuous mode, a rapid temperature increase is observed, reaching approximately 1 °C within 60 seconds, particularly crossing the hyperthermia threshold. This protocol was selected for cell sonication. In contrast, in the case of the 40% pulsed mode, a more moderate temperature elevation across all intensities was demonstrated, which confirmed its suitability for minimizing thermal effects during ultrasonic exposure.

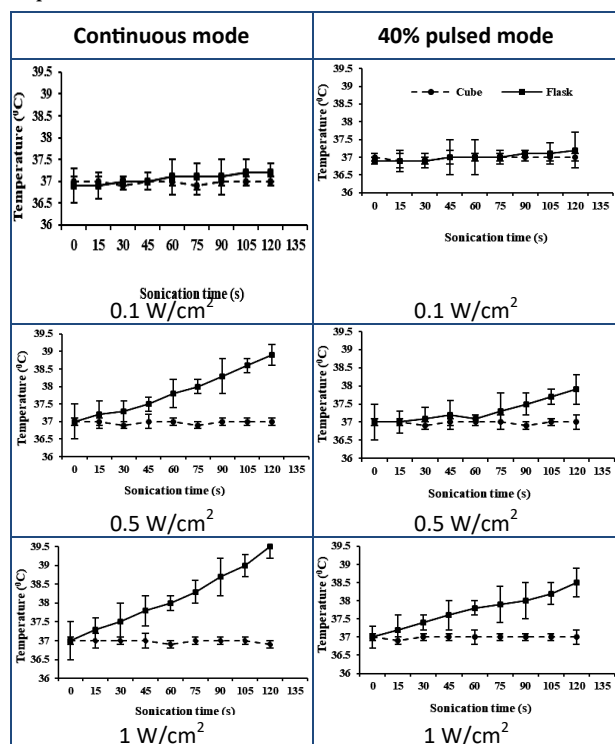


Figure 3. Temperature monitoring diagrams of the cell flask exposed to 1 MHz ultrasonic waves in both continuous and 40% pulsed modes at intensities of 0.1, 0.5, and 1 W/cm². Data are presented as mean $\pm$ standard deviation ($n = 5$).

It should be noted that the error bars in the graphs represent the standard deviation (SD) of the data. Each data point is based on five independent experimental repetitions ($n = 5$) to ensure the reliability and reproducibility of the temperature

measurements under each sonication condition.

Toxicity of gold NPs on MCF-7 and MDA-MB-231 cells

To assess the cytotoxicity of artificially produced NPs, various concentrations of NPs were cultured alongside MCF-7 and MDA-MB-231 cells over a duration of 48 hours in a laboratory setting. It is important to note that the cells in the control cohorts, which were devoid of gold NPs, were also maintained under the same experimental conditions concurrently. Following the incubation period, the cellular viability was determined by quantifying the mitochondrial activity using the MTT assay across all experimental groups. The outcomes derived from carrying out five independent replicates have been meticulously tabulated, illustrating the mean values along with the corresponding standard deviations in figure 4.

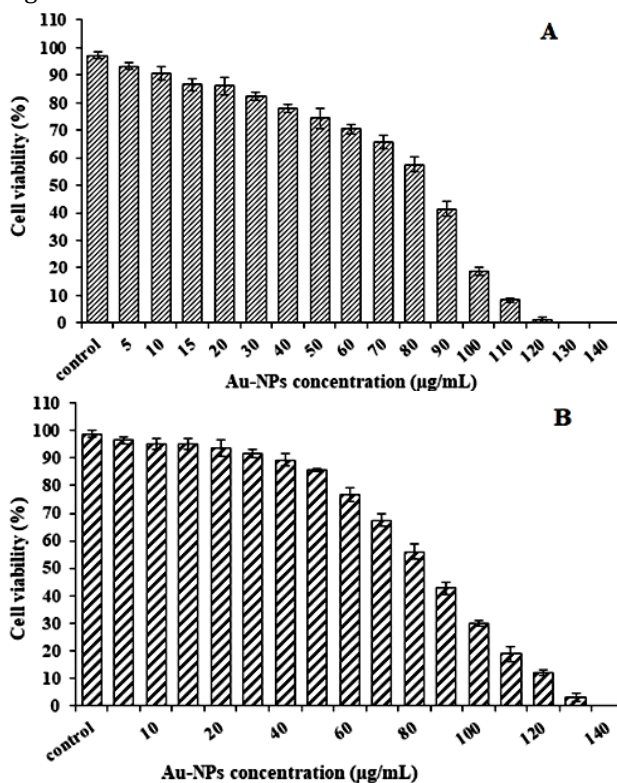


Figure 4. Mean and standard deviation of normalized optical density as a measure of cell viability of MCF-7 (a) and MDA-MB-231 (b) cells at different concentrations of gold NPs.

Based on the data visualized in the aforementioned figure 4, it is discernible that when exposed to a concentration level of 50 µg/ml, the MCF-7 cell line exhibited an approximate survival rate of 90%, whereas the MDA-MB-231 cell line demonstrated a survival rate of 80%. The resultant effects observed are deemed favorable and statistically significant at a significance level of 0.05.

Sonication of cell lines in the presence and absence of selected gold NPs

To ascertain the enhancement in therapeutic

efficacy, or more precisely to assess the sonosensitization of gold NPs, the designated concentration of gold NPs underwent an incubation period lasting 48 hours with MCF-7 and MDA-MB-231 cells within each culture flask, maintaining a fluence level of approximately 80%. Subsequent to this incubation period, the culture medium surrounding the cells in every well was carefully extracted and subjected to two rounds of washing with a PBS solution in order to eliminate any residual gold NPs present in the culture medium, thus priming the cells for irradiation treatment.

Concurrently, in addition to the experimental groups receiving sonication therapy either in isolation (figure 5) or in conjunction with gold NPs (figure 6), control groups (comprising solely of cells) were also cultured alongside. Following a 48-hour irradiation period, the viability of the cells in all groups was assessed using the MTT assay, with the recorded outcomes being subsequently averaged. The data obtained from five replicates are visually represented in Figures 5 and 6 for comprehensive analysis and comparison purposes.

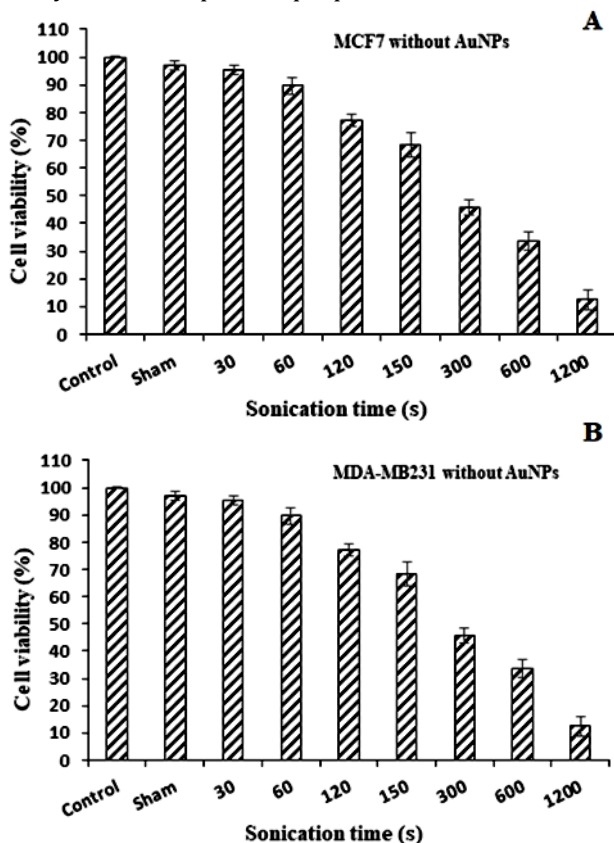


Figure 5. The mean and standard deviation of normalized optical density as a measure of cell viability of MCF-7 (a) and MDA-MB-231 (b) cells in the absence of gold NPs at different sonication times. Data are presented as mean $\pm$ standard deviation (n = 5).

Upon meticulous examination of figures 5 and 6, it can be deduced that the application of ultrasound waves in conjunction with gold NPs leads to a higher degree of cytotoxicity compared to scenarios where

NPs are absent (statistically significant at $p < 0.05$). As depicted in figures 5 and 6, the survival rate of cells exhibited a declining trend with prolonged exposure to irradiation across all three irradiation conditions under study. In fact, gold NPs can be used as a co-factor in other common treatment methods. In this study, the effective role of gold NPs along with other combined treatments is suggested as an effective factor in the death of breast cancer cells and improving the current treatment process.

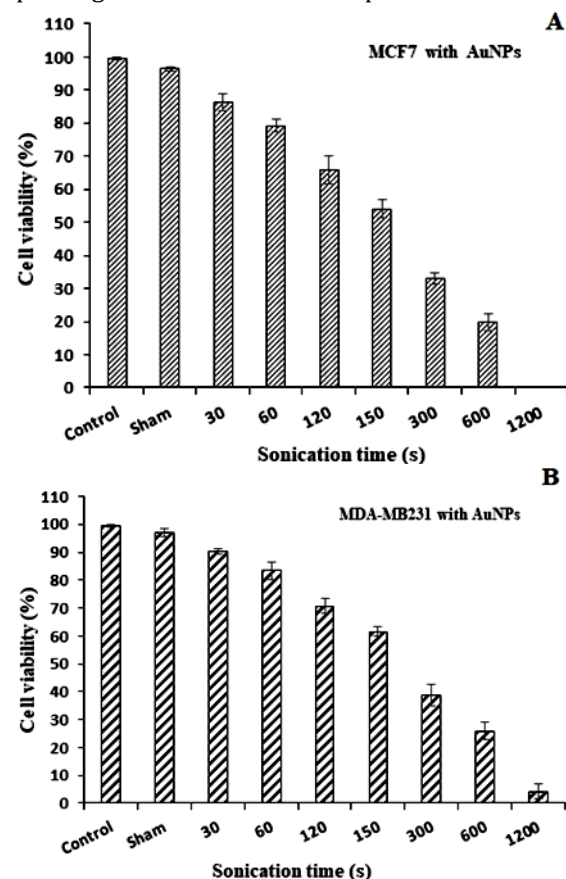


Figure 6. The mean and standard deviation of normalized optical density as a measure of cell viability of MCF-7 (a) and MDA-MB-231 (b) cells in the presence of gold NPs with selected concentrations at different sonication times. Data are presented as mean $\pm$ standard deviation (n = 5).

DISCUSSION

Breast cancer is currently the most frequently diagnosed cancer globally, with its incidence closely linked to human development. It is anticipated that the number of cases will rise notably in regions undergoing economic transitions. Conversely, survival rates in less developed regions are notably lower. Disparities in global survival rates stem from various factors, including diagnostic delays and inadequate treatment options (18). In various studies, the toxicity effect of gold NPs on healthy and cancer cells has been studied. The researchers' results show that the toxicity mechanism of these NPs is through

the production of active oxygen^(13,14). An increase in the amount of active oxygen causes the DNA molecule to break and eventually leads to cell death⁽¹⁹⁾. By increasing the concentration of gold NPs, the amount of NP accumulation in the cell increases and causes a decrease in mitochondrial function and an increase in cell death. Due to their small size, NPs can accumulate in cancer cells⁽²⁰⁾. Therefore, it is necessary to choose the optimal concentration of NPs, because more NPs cause more toxicity to the human body. Consequently, in this study, we tried to select the optimal concentration of gold NPs *in vitro* experiments and the optimal dose of ultrasound waves in the treatment of two cell lines of breast cancer.

Based on the observations and results of the study by Song and his colleagues⁽²¹⁾, it was shown that HeLa and MCF-7 cells absorb 57 nm NPs more than 84 nm NPs. This result is almost consistent with the results of Chithrani *et al.*'s studies⁽²²⁾, which showed that 50 nm is a good choice for gold NPs in the treatment of tumor cancer cells. Based on these results, the size of NPs has an effect on biomedical applications. In addition, a study conducted by Song *et al.*⁽²¹⁾ showed that the cellular uptake of gold NPs for MCF-7 cells is approximately 10 times higher than that of HeLa cells. Some studies have shown that the use of gold NPs to treat cancer cells does not affect on normal cells⁽²³⁾. In a study, Cho and his colleagues⁽²⁴⁾ showed that if the size and concentration of gold NPs are appropriate, they do not have a toxic effect on cells. They showed that the use of gold NPs with a size of 10 nm does not have a toxic effect on the cells and by absorbing laser light, they heat, and destroy cancer cells. Also, in a study, HeLa and T3/NIH3 cells were exposed to gold NPs with dimensions of 20 nm for 3 hours, which resulted in cell survival of 80% and 95%, respectively⁽²⁵⁾. It is also possible to refer to the study of Moradi and his colleagues in 2019⁽²⁰⁾, in which the toxicity effect of gold NPs synthesized by the Turkovich method and with an approximate diameter of 60 nm on Y79 cells of retinoblastoma eye tumor was investigated. Moradi *et al.*^(26, 27) demonstrated that induced retinoblastoma tumor volumes in rabbit eyes significantly decreased after sonication followed by GNPs injection ($P < 0.05$). Histopathologic studies confirmed the necrosis of viable retinoblastoma cells.

The study found that living cell percentages decreased with higher concentrations of gold nanoparticles (NPs), showing a significant difference ($P < 0.05$) at levels exceeding 69.1 micrograms per milliliter. At 69.1 micrograms per milliliter, cell survival (25%) was similar to that of the control group ($P > 0.05$). The cell viability after 48 hours using the MTT test ranged from 53%-75%, aligning with previous studies but with a different cell line and gold NPs of 14 nm diameter. This research explored the effects of gold NPs on MCF-7 and MDA-MB-231

cells, identifying 50 µg/ml as a suitable concentration, resulting in about 80% living cells, indicating minimal toxicity. The findings support the use of lower doses of gold NPs in cancer treatment to reduce side effects while potentially enhancing the efficacy of existing therapies. The study suggests that gold NPs could serve as co-factors in cancer treatments and improve the overall treatment process for breast cancer.

CONCLUSION

It is concluded that there is a sonosensitizing effect related to the gold NPs. Combined with gold NPs, the survival rate of cells decreased with prolonged ultrasound exposure. In addition, the obtained results suggested that gold NPs, considering optimal size and concentration, have great performance as effective sonosensitizers that can be used to enhance the therapeutic efficacy of ultrasound-based treatments. Furthermore, this combined approach introduced itself as a promising method for reducing the side effects associated with conventional chemotherapy or radiotherapy, without compromising treatment effectiveness.

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Author contribution: J.O., conducted the experiments, analyzed the data, and prepared the initial draft of the manuscript. M.M.D., supervised the research process, contributed to data interpretation, and revised the manuscript critically for important intellectual content. H.M. and S.R.M., as advisors of the project, were involved in data analyses and interpretations. All authors read and approved the final version of the manuscript.

Availability of data and material: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Ethical approval: This article does not contain any studies with human or animal subjects performed by any of the authors. The study complied with the principles of the *Declaration of Helsinki*.

Possible AI usage: There is no reportable usage of AI applications.

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