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Induction of a bystander effect after therapeutic ultrasound exposure in human melanoma: *In-vitro* assay

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► ***Original article***

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ABSTRACT

Background*:** The induction of bystander effect via ionizing radiation has been well proven. However, few studies have investigated the bystander effect following non-ionizing radiation, such as ultrasound waves. Here, the bystander effect after different sonication times on human melanoma cell line (A375), is evaluated by assessing cell viability and apoptosis. ***Materials and Methods*:** The cells were divided into two main target and bystander groups. Target cells were exposed to 1 MHz ultrasound at 2 W/cm² intensity for 1, 2, 5 and 10 min with an ultrasound unit. Then, bystander cells received the cell culture medium of target cells. MTT and flow cytometry assays were used to determine the cell' viability at different times after exposure and medium transfer, as well as the detection of apoptosis. ***Results: The cell' viability in ultrasound-exposed target cells was less than 75% for 24 and 48 h incubation. Furthermore, bystander cell' viability was not significantly different from the control group 1 and 12 h after receiving the culture medium of target cells. However, bystander cells viability 24 and 48 h after target cells medium transfer was significantly decreased (P=0.01). The apoptosis rate of bystander cells, 24 and 48 h after receiving the cell culture medium of target cells, showed significant differences from the control group. ***Conclusion:*** This research results revealed that the ultrasound waves could induce a biological effect in A375 bystander cells which were not directly exposed to direct ultrasound.

Keywords: *Bystander effect, ultrasound waves, MTT assay, apoptosis assay, A375 cell line.*

INTRODUCTION

It is well established that radiation can induce cellular responses in non-irradiated cells which are termed radiation-induced bystander effect (RIBE) [(1)]. This phenomenon leads to radiation induced-injuries in non-irradiated bystander cells. As reported, non-irradiated cells show a variety of biological responses, including reduced viability fraction [(2)], chromosomal instability [(3)], DNA double strand-breaks, apoptosis [(4)], and changed dynamic balance between proliferation [(5)].

RIBEs, after ionizing radiation, have been well documented. However, a few studies have investigated the bystander effects as a result of other environmental factors, including some electromagnetic waves as ultraviolet light [(6–10)] and radio frequency waves [(11)] and also after chemotherapy [(12)] and photodynamic therapy,

treatment modalities [13–15]. Bystander mechanisms have not fully been understood yet. However, it has been found that some signaling factors, such as Reactive Oxygen Species (ROS), NO [15], and OH radicals [17,18], released from irradiated cells have medium-mediated bystander effect.

Ultrasound is one of the non-invasive modalities for the treatment of some cancers [19], which is defined as mechanical vibration produced via sound waves with a frequency of more than 20 kHz [20]. It has been reported that the effective therapeutic frequency of ultrasound is in the range of 0.8 to 3 MHz [21,22] .Ultrasound waves by different thermal, mechanical, and micro-bubbles cavitation can play vital roles in cancer treatments [23–25]. Physically, the ultrasound waves could may increase the temperature of the cell microenvironment by the generation of thermal energy [22,26,27] and also bubble [28]. Ultrasound, with a frequency of 1 MHz and 0.5 to 3.0 W/cm^2 intensities (low-intensity ultrasound), can produce inertial cavitation inside the tumor volume [19]. Mechanical shock waves induce biological effect as a result of the collapse in microbubbles. In other words, when the bubbles burst by localized spots with high temperature and pressure, they cause free radicals formation and apoptotic initiators, that inhibit cancer cell growth [19]. Hydroxyl radicals (·OH), hydrogen peroxide (H_2O_2), and hydroperoxyl radicals (HOO) are some species of produced free radicals by ultrasound waves, which induce chemical changes [27]. Apoptosis is the endogenous programmed cell death that allows the maintenance of cellular homeostasis [29]. Based on the author's knowledge, in most biological studies, apoptosis induced by ultrasound even at low intensities in target cells has been proven [30], and hence there is no information to describe the induced apoptosis in bystander cells.

To the best of the author's knowledge, there is no study on the bystander effect following ultrasound therapy. Therefore, considering the therapeutic effects of low-intensity ultrasound and also according to the role of the bystander effect on cancer treatment, in this work the bystander effect following ultrasound exposure on the A375 cell lines are investigated. To evaluate the effects of target cell following ultrasound exposure on the bystander cells, the cell viability and apoptosis are evaluated using MTT assay and flow cytometry, respectively.

MATERIALS AND METHODS

All procedures performed in this study involving cellular experiments (in vitro), were under the ethical standards of the institutional and/or national research committee and the 1964 Helsinki declaration, as well as its later amendments or comparable ethical standards.

Cell line and culture conditions

A375, as a cancer cell line of human melanoma, was purchased from Pasteur Institute in Tehran, Iran. The cells were cultured in Roswell Park Memorial Institute1640 medium (RPMI 1640, Gibco, Germany) containing 10% Fetal Bovine Serum (FBS, Gibco, Germany), streptomycin (1 mg/ml, Biosera, France), and penicillin (1000 units/ml, Biosera, France); then, they were incubated at 37 °C in a humidified atmosphere with 5% CO_2. The cells were cultured in T25 flasks and then transferred to 12 and 96 wells plates for examinations.

Ultrasound generator system and exposure set up

In this study, ultrasound irradiation was provided by a therapeutic ultrasound unit (215A; a coproduct of Novin Medical Engineering Co., Tehran, Iran; and EMS Co., Reading, Berkshire, England) in continuous mode at a frequency of 1 MHz with an intensity of 2 W/cm^2 (I_{SATA}). Acoustic calibration for the frequency and intensity of the device was carried out in degassed water in a tank, using a calibrated PVDF-type hydrophone (PA124, Precision Acoustics Ltd., Dorchester, Dorset, UK) on the transducer axis to record the ultrasonic signals. The temperature rise during the exposure was checked by a thermometer, which was inserted in the water tank, to keep the temperature of the cells below the hyperthermia

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level.

For ultrasound exposure, the transducer was fixed in a hole at the bottom of the water tank; then, the 12-well plate was placed in the near field of the transducer. The floor of the plate was in direct contact with the water in the water tank.

Cell viability assay

The MTT test was used to determine the percentage of cell viability. After cell treatment, the cell culture media was removed, and the culture medium was replaced with a mixture of 100 µl RPMI and 10 µl of MTT solution (5 mg/mL) (Sigma, St. Louis, MO, USA) for each well (in a 96-well plate) then incubated for 4 hours. Then, the MTT solution was removed entirely, and 50 µl of Dimethyl Sulfoxide (DMSO, Sigma, USA) was added and incubated for 15 min. All experiments were repeated for three independent times. Finally, optical densities (OD) were measured at 570 nm by a spectrophotometer (Stat Fax 2100, USA) .Cell viability values were determined using equation (1):

$$\text{Cell viability} = \frac{OD_{treated}}{OD_{control}} \times 100 \quad (1)$$

Ultrasound exposure of target cells

To determine the effect of ultrasound waves at different times on target A375 cells, the cells were cultured in 12-well plates (5×10^4 cell/well). After the cells adhered to the plate, the cell culture was replaced with fresh medium containing 10 % FBS and exposed to 1 MHz ultrasound at 2 W/cm^2 intensity for 1, 2, 5, and 10 min with ultrasound unit. Then, the viability of target cells was measured 1, 12, 24, and 48 h after ultrasound exposed with the MTT test.

Induced bystander effect using medium transfer

In this study, the medium transfer method was used to induce a bystander effect in A375 non-irradiated cells. One hour following ultrasound exposure, cell culture of ultrasound exposed cells was collected and passed through a 0.22 µm filter (Orange Scientific, Belgium) to remove cells plus cell debris and transfer to the specified bystander cell plates. The bystander cells were incubated for 1, 12, 24, and 48 hours after receiving the target cell culture medium. Then, MTT assay and apoptosis tests were performed .

Measurement of bystander cells apoptosis

Apoptosis was detected by the eBioscience™ Annexin V Apoptosis Detection Kit II (Invitrogen, USA). After bystander cell treatment, the culture medium of each well containing floating cells was collected, and adherent cells were detached by trypsin. Afterward, both floating and adherent cells were poured into a flow cytometry tube, washed twice with PBS (100x), and centrifuged at 300g for 10 minutes at 15 °C. Then, the cell was incubated in 100 µl of binding buffer (1x) with 2 µl Annexin-V and 2 µl propidium iodide (PI) for 15 min in the dark at room temperature. Next, 10,000 cells, for each sample were recorded on a Flow Cytometer (BD FACS Calibur, USA), and fluorescence-activated cell sorting (FACS) data were analyzed using open-source software.

Data analysis

Each experiment was carried out in triplicate. The viability data were noted as mean ± standard deviation. The data distribution was normal according to the Kolmogorov-Smirnov normality test, and the statistical comparison of groups was done by one-way analysis of variance (ANOVA) at $P < 0.05$. Each experiment was performed in triplicate.

RESULTS

The data obtained in the first part of the study permit comparing the viability of A375 cells in the target groups (ultrasound exposed cells) 1, 2, 5, and 10 min with respect to the control cells following incubation times of 1, 12, 24, and 48 h (figure 1).

The graphs indicate the dependence of the range value of relative cell viability on the incubation and ultrasound exposure time. The

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graph of 1 min shows large variability in the viability values for each exposure time. The results of the 12 h experiment show a statistical difference for 5 and 10 min ultrasound exposure time; the lowest viability value is found for 10 min from all exposure times (90%).

The graph of the 24 h illustrates a decrease in viability of ultrasound exposed groups at 1, 2, 5, and 10 min. The experimental results of the 48 h show the statistical difference ($P < 0.001$) between ultrasound exposed groups at 1, 2, 5, and 10 min with the control group (table 1), as the 24 h group. The lowest viability value is found for 10 min of ultrasound exposure time, which reached values of less than 73% of the control group. The group 5 min exposure time is on the viability level of 85% value of the control. The group 1 min ultrasound exposure time reaches the higher value of viability in comparison with the other groups, about 97% value of the control group.

According to figure 2, there are no differences in the viability of bystander groups with 1 and 12 h incubation for all exposure times. The graphs exhibit similar levels of bystander cell viability after 1 and 12 h incubation for all exposure time to the control group ($P > 0.05$). However, the viability of bystander groups after 24 and 48 h incubation times following 10 min ultrasound exposure shows the statistically significant difference with the control group ($P < 0.001$), in these groups the viability level of 90% value of the control. In these groups, they are more apoptotic than the control group.

Moreover, the dual staining method with Annexin V-FITC and PI was utilized to assess apoptosis in bystander cells. Figure 3 illustrates the fraction of A375 bystander cells undergoing apoptosis. The sum of the lower right quadrant (early apoptosis) and upper right quadrant (late apoptosis) represents the total apoptosis rate. In this study, apoptosis induction in bystander cells after 24h (10.94%) and 48h (13.25%) incubation with target cell culture media exposed with 10 min ultrasound is evaluated. A slight increase in the apoptosis of bystander cells after 24 h incubation compared to the control group is observed ($P = 0.01$), and the significant difference occurs at 48 h incubation after 10 min ultrasound exposure. Therefore, 10 min ultrasound exposure time with 2 W/cm^2 intensity induces apoptosis in bystander cells compared to control cells.

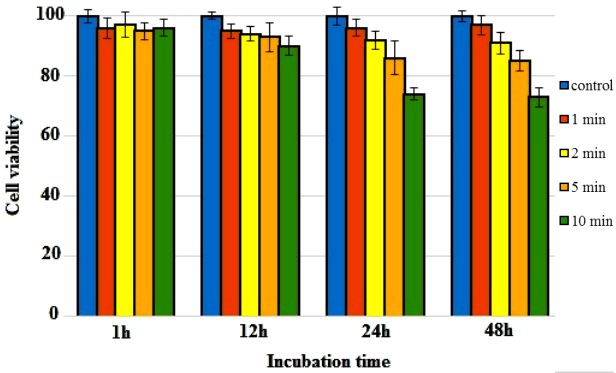

Figure 1. Cell viability in A375 target cells after different ultrasound exposure (1, 2, 5, and 10 min) and incubation times (1, 12, 24, and 48 h).

Table 1. Significance of differences in viability values in target and bystander groups. Target cells are exposed to ultrasound of intensity 2 W/cm2 for 1, 2, 5, and 10 minutes. Then the medium transfer technique is performed. Cell viability is evaluated after 1, 12, 24, and 48h incubation times. ■ no significance, ▲statistical significance at $P < 0.05$.

Target		Bystander	
	Control		Control
1h		1h	
Con	----	Con	----
1min	■	1min	■
2min	■	2min	■
5min	■	5min	■
10min	■	10min	■
12h		12h	
Con	----	Con	----
1min	■	1min	■
2min	■	2min	■
5min (93%)	▲	5min	■
10min(90%)	▲	10min	■
24h		24h	
Con	----	Con	
1min	■	1min	■
2min(92%)	▲	2min	■
5min(86%)	▲	5min	■
10min(74%)	▲	10min (89%)	▲
48h		48h	
Con	----	Con	----
1min	■	1min	■
2min(91%)	▲	2min	■
5min(85%)	▲	5min	■
10min(73%)	▲	10min(86%)	▲

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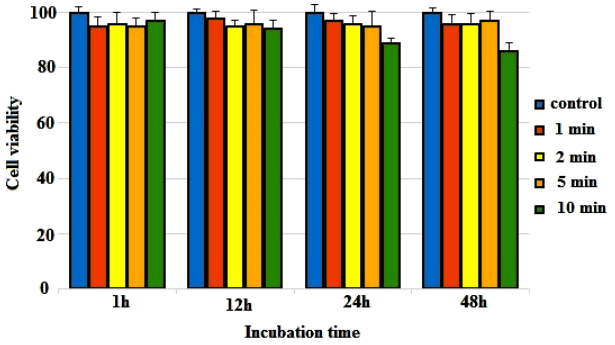

Figure 2. Cell viability in A375 bystander cells after different ultrasound exposure (1, 2, 5, and 10 min) and incubation times (1, 12, 24, and 48 h).

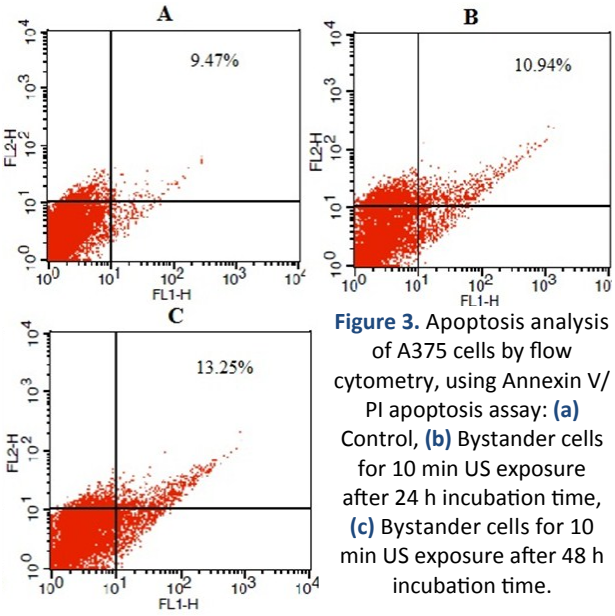

Figure 3. Apoptosis analysis of A375 cells by flow cytometry, using Annexin V/PI apoptosis assay: **(a)** Control, **(b)** Bystander cells for 10 min US exposure after 24 h incubation time, **(c)** Bystander cells for 10 min US exposure after 48 h incubation time.

DISCUSSION

Studies have shown that the therapeutic frequency of ultrasound can induce cell death. Under our conditions, cell death is detected by the MTT method in target and bystander cells. In the present paper, the MTT assay results of target cells indicate therapeutic ultrasound with 1 MHz frequency, and 2 W/cm^2 intensity causes A375 cell death.

The cell death induced by ultrasound is predicted to be caused by acoustic cavitation. These findings are in agreement with the presented results based on which the percentage cell death of lung cancer of cell lines after ultrasound exposure has been revealed to be 32% [31]. Also, in other studies [31,32], cell death by ultrasound in target cells have been from 55% to 68%. In another study, Wang et al. showed that, in 2 W/cm^2 ultrasound intensity, the acoustic cavitation becomes a dominant effect as a result of producing more •OH free radical, which may cause cell death [33]. Based on the results, the decline in cell death has been enhanced by the increased exposure time (figure 1).

Moreover, the cell death for 10 min ultrasound exposure is significantly higher than 1, 2, and 5 min. In some studies with different cell lines, it is demonstrated that cell death of ultrasound exposed cells increases, and it is mainly dependent on time. Therefore, cell viability is reduced with increasing ultrasound exposure time [34,35].

In this study, the cell viability of bystander groups at different irradiation and incubation times is evaluated. The cell viability of bystander groups illustrates a significant difference compared to the control group, when both ultrasound exposure and incubation times are increased.

The bystander cells after 24 and 48 h incubation with 10 min ultrasound exposed target cell media show significant cell death. However, this phenomenon does not occur in bystander cells after less than 10 min of ultrasound exposure time and also 1 and 12 h incubation times. Therefore, it seems that, increasing the exposure time is a vital factor to induce bystander effect. It is due to the high free radical production when ultrasound exposure time increases. Also, cell viability after 24 and 48 h incubation is not significantly different from each other. Therefore, 24 hours can be a suitable incubation time to induce the bystander effect in non-targeted cells.

Many studies have shown that ionizing radiation can induce cell death (23±2%) in non-irradiated cells even after 30 min incubation bystander cells with target cell culture media [36]. However, in this study, cell death is observed in the bystander group in 24 h after receiving the target group medium. These findings can be related to this fact that ionizing radiation has a destructive nature and different

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mechanisms to induce bystander effect compared with ultrasound waves.

It is evidently that the viable cells in bystander cells decrease after receiving the target culture medium. The mechanism of cell death induced by ultrasound in A375 bystander cells is evaluated by flow cytometry. The results indicate that the culture media of ultrasound exposed target cells after 10 min can induce apoptosis in the bystander cells after 24 and 48 h incubation (figure 3). The percentage of early apoptosis is increased ($P<0.05$) compared to the control cells, which have proved the apoptosis of cells to be induced in bystander cells by ultrasound indeed.

There was no information regarding the influence of ultrasound exposed target cells on bystander apoptosis rate. Whereas previous studies have shown increased apoptotic cells after ultrasound exposure in target cells [29], based on the author's our knowledge, apoptosis induction in bystander cells has not been investigated in other studies. However, further evaluations, such as analysis of protein expression changes and their functions that are crucial to detect the related metabolic process and assessment of the potential mechanism of ultrasound that may cause cell apoptosis in bystander cells, are needed.

In conclusion, recent evidence indicates that ultrasound has good prospects in the treatment of melanoma. In this study, the low intensity of ultrasound waves is utilized to more accurately evaluating them on A375 human melanoma cells, which are located outside the radiation field. According to the results of this study, the killing/destructive effect of ultrasound on A375 cells is exposure time-dependence. Also, ultrasound waves can create signals in the culture medium of directly exposed cells that may lead to apoptosis in bystander cells. Therefore, ultrasound waves can affect the cells outside the ultrasound exposure field, as a result of bystander effects.

CONCLUSION

In this research, the findings prove that the killing effect of ultrasound on A375 cells is exposure time-dependence. Also, ultrasound waves can create signals in the culture medium of directly exposed cells that may lead to apoptosis in bystander cells. Therefore, ultrasound waves can affect the cells outside the ultrasound exposure field, as a result of bystander effects. In other words, the biological effects of ultrasound waves are not limited to the physical exposure field.

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Ethical statements:

This article does not contain any studies on human or animal subjects performed by the any of the authors.

***Conflicts of interest*:** *Declared none.*

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