Bystander Effect of Therapeutic Ultrasound in the Presence of Cisplatin: An in Vitro Study on Human Melanoma Cells

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ABSTRACT

Background: In the bystander effect, non-irradiated cells receive biological signals from adjacent irradiated cells and undergo a variety of alterations, considered recently in non-ionizing irradiation like ultrasound waves. In this study, the bystander effect of therapeutic ultrasound exposure alone and in combination with cisplatin was determined.

Objective: This study aims to determine the bystander effect caused by ultrasound and cisplatin.

Material and Methods: This experimental study was conducted on the human melanoma cell line including two groups of target and bystander cells. The target cell group was divided into three sub-groups of ultrasound irradiation alone, cisplatin alone, and ultrasound irradiation in the presence of cisplatin that the culture medium of these three groups of cells was transferred to the bystander cell group using the medium transfer technique. Then, apoptotic bystander cells and the expression of P53 and HO-1 in target and bystander groups were measured.

Results: The results of the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and apoptosis assay showed that cell death in target and bystander groups receiving the ultrasound with cisplatin is higher than in the ultrasound without cisplatin. PCR (the polymerase chain reaction) results in the target and bystander groups receiving treatments with increased expression of the P53 gene. Target and bystander groups receiving the ultrasound without cisplatin showed a decrease in HO-1 gene expression, while the ultrasound with cisplatin showed an increase in the HO-1 gene compared to the control group.

Conclusion: Combining ultrasound with ultrasound and without it can transfer bystander signals to the cells that are not directly treated.

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Keywords

Bystander Effect; Ultrasonic Therapy; Cisplatin; Melanoma; Apoptosis

Introduction

Integrated communication mechanisms between the cells including normal and cancerous cells are essential for responding to both external and internal stimuli [1]. The bystander effect is one of these intercellular communications with an important role in the radiobiology and cancer treatment realm [2]. In this phenomenon, non-irradiated cells located in the region of interest receive biological signals from adjacent irradiated cells and undergo a variety of alterations [3] such as genomic instability [4], mutation, and apoptosis [5].

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Although the exact mechanism of the bystander effect is not yet fully discovered, some signaling pathways such as gap junction intercellular communications or releasing some factors like reactive oxygen species (ROS), nitric oxide, and hydroxyl (OH) radicals from irradiated cells are involved in generating this effect [6, 7].

Many studies show the potential of ionizing radiations such as X and gamma rays [8] or even chemotherapy agents [9] for inducing the bystander effect but a few studies investigated safer cancer treatment modalities such as therapeutic ultrasound (US) in creating this effect [10, 11].

Using ultrasonic waves because of their non-ionizing nature is considered in medicine for either diagnostic or treatment applications [12].

The phenomenon known as 'acoustic cavitation' plays the main role in the treatment with ultrasound waves [13]. Extreme temperature and pressure resulting from acoustic cavitation can not only damage the cell membrane or facilitate drug delivery in the sonoporation effect but also induce phenomena like the bystander effect by generating free radicals such as reactive oxygen species, OH, hydrogen peroxide, and hydroperoxyl [14, 15].

Due to the limited therapeutic effects and unavoidable side effects of conventional cancer treatment modalities such as systematic toxicity of chemotherapy, combinational treatment approaches are more favorable [16, 17]. Based on some studies conducted on the concomitant use of cisplatin as a chemotherapy agent with ultrasonic exposure for the US-mediated chemotherapy treatment, it was more efficient than cisplatin lonely [18, 19].

No studies have been conducted on the bystander effect as a result of ultrasound exposure combined with chemotherapy agents [20, 21]. Therefore, the aim and the novelty of the present study are to evaluate the contribution of the US in the presence of cisplatin to induce the bystander effect, and also this study was conducted on melanoma as the deadliest type of skin cancer with resistance to conventional therapies such as chemotherapy.

Material and Methods

Cell culture

Based on the aim of this experimental study, the A375 melanoma cell line was purchased from the Pasteur Institute of Tehran, Iran, cultured firstly in T75 flasks in the RPMI 1640 medium (Gibco, Germany) comprising 10% FBS (Gibco, Germany), and 5% Pen-Strep (Biosera, France), and secondly incubated at 37 °C in a humidified atmosphere with 5% CO_2 and then transferred to plates (12 and 96-well) for examinations.

US wave generator and condition configuration for US exposure

The 1 MHz therapeutic US unit (215X; a co-product of Novin Medical Engineering Company, Tehran, Iran and EMS Company, Reading, Berkshire, England) in a continuous mode and probe with a 7.0 cm^2 effective radiation area and a 29.8 mm diameter was used for experiments on the cells. For ultrasound irradiation, the unfocused transducer was placed at the bottom of a container filled with water (ultrasound bath). Target plates (12-well) were irradiated in the field near the transducer such that the distance between the cells adhered to the floor of the plate and the US transducer was 15 cm (as shown in Figure 1).

Cell viability assay

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was firstly used to evaluate the survival percentage of the cells and secondly, the cell culture medium was replaced with a blend of 90 microliters of RPMI and 10 microliters of the MTT solution (5 mg/mL; Sigma, St. Louis, MO, USA) for each well (in a 96-well plate) and incubated for 4 h after the treatments the cells. Next, the solution was entirely removed and replaced

Figure 1: Ultrasound exposure setup configuration.

with 50 microliters of DMSO (Dimethyl sulfoxide) (Sigma, USA) and incubated for 15 min. The viability percentage of the treated cells was calculated while considering the control group.

Evaluation of cisplatin cytotoxicity

To define the optimum concentration of cisplatin, 2×10^3 cells/wells were cultured in a 96-well plate, and after 24 h, the culture medium was replaced with a fresh cell culture having various concentrations of cisplatin (Mylan, 1mg/ml, Franc) including 1, 5, 10, 20 and 30 µM. After incubation for 24 h, cell viability was measured by the MTT assay.

Medium transfer technique to induce the bystander effect

The medium transfer technique was employed to induce the bystander effect on the cells that were not exposed to any treatment. To determine the effect of US waves alone and in combination with cisplatin on target A375 cells, the cells were cultured in plates (12 wells) (5×10^4 cells/well). After 24 h, the cells were treated with cisplatin (5 µM) for 3 h. Then, the culture medium was removed and the cells were washed twice with PBS and exposed to 1 MHz US waves at 2 W/cm^2 intensity (ISATA) for 10 min. The cell culture medium was extracted an hour after US irradiation and purified with a 0.22 µm filter (Orange Scientific, Belgium) to remove cells and cell debris. Next, non-exposed bystander cells received a filtered cell culture medium and further examinations were performed after 24 h of incubation. Eventually, the cell viability of target cells was measured using the MTT test after 24 h of incubation.

Apoptosis measurement in bystander cells

The apoptotic percentage in the bystander cells was evaluated by the eBioscience™ Annexin V Apoptosis Detection Kit II (Invitrogen, USA) and flow cytometry. After bystander cell treatment, cells were extracted from the culture medium and incubated in 100 µL of the binding buffer (1x) comprising 2 µL Annexin-V and 2 µL propidium iodide (PI) for 15 min in a dark place with ambient temperature. Then, 10^4 cells for each sample were analyzed with a flow cytometry machine.

RNA extraction and Real-time PCR

Entire RNA (Ribonucleic acid) from cells using the Biofact Total RNA prep Kit (BioFACT, Korea) according to the manufacturer's commands. The quantity and quality of isolated RNA were specified by assessing absorbance at 260 nm and 280 nm with NanoDrop™ 2000 Spectrophotometer (Thermo Scientific). To avoid potential contaminations with genomic DNA (deoxyribonucleic acid), the RNA samples were treated with DNase I (Thermo Scientific). Five micrograms of total isolated RNA were employed for synthesizing cDNA using BioFACT X Onestep Real-time PCR (the polymerase chain reaction) Master Mix kit (BioFACT, Korea), and oligo (dT) primers. The primers were used for all assayed genes. The real-time polymerase chain reaction was performed using BioFACT 2X Real-time PCR Master Mix (High ROX) containing SYBR Green (BioFACT, Korea) and the StepOne Plus™ Real-time PCR detection system (Applied Biosystems, Foster City, CA, USA) (Table 1). Moreover, GAPDH was used as an endogenous control. Table 1 shows the primers used in the detection of the different genes.

Data analysis

Statistical analysis was conducted using the SPSS software package (version 22.0). According to the Kolmogorov-Smirnov normality test, the data distribution was normal. One-way ANOVA, Tukey's multiple comparisons, and Dunnett's tests were used at $P<0.05$, and each experiment was performed in triplicate.

Results

Recognition of the optimum concentration of cisplatin

According to Figure 2, the optimal concentration of cisplatin required for the experiments

Table 1: Sequences (5′ to 3′) of the primers used in the detection of the different genes.

Gene	Forward	Reverse
GAPDH	TGGTATCGTGGAAGGACTC	AGTAGAGGCAGGGATGATG
P53	TCTGACTGTACCACCATCCACTA	CAAAACGCACCTCAAAGC
HO-1	CAACAAAGTGCAAGATTCTG	AAAGCCCTACAGCAACTG

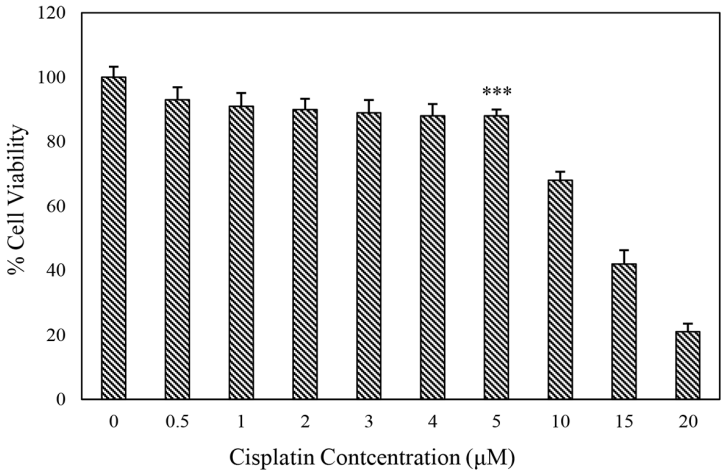

Figure 2: Viability of the cells in the presence of cisplatin with different concentrations. Data are expressed as mean±SD (standard deviation) (n=3) in comparison with the control group. *Significant difference in the comparison with the control group (*$P<0.05$,**$P<0.01$,***$P<0.001$).

with cell viability of 88±2% in the A375 cell line is 5 µM (P<0.001). For concentrations greater than 5 µM, the rate of cell death is extremely high and causes toxicity.

Effect of US irradiation alone and in combination with cisplatin on the target and bystander cells

According to data in Figure 3, the percentage of cell viability in target cells was 27 and 61% for cells that were directly exposed to US waves in the presence and absence of cisplatin, respectively, showing a significant difference in the percentage of cell viability between the target cells and control groups (P<0.001).

As shown in Figure 2, cell viability in the bystander cells causes increased cell death in the presence and absence of cisplatin for 2 W/cm^2 US intensity 24 h after medium transfer. The percentage of cell viability in bystander cells by the US in the presence and absence of cisplatin is 77 and 82%, respectively. The statistical comparison of these results demonstrates a significant difference in the percentage of cell viability between bystander cells and control groups (P<0.05).

The lower and upper right quadrants show early and late apoptosis, respectively (Figure 4). The percentage of apoptosis after the exposure to US wave with or without cisplatin in the target groups is 63.98 and 25.48%, and in bystander groups is 13.55 and 10.94%, respectively.

Based on Figure 5, the percentage of apoptotic cells in bystander groups after US waves exposure to target cells in the presence and absence of cisplatin significantly differs from the control group (P<0.001).

Expression levels of P53 and HO-1 genes in target and bystander cells

According to Figure 6, the P53 gene expression in US exposure without cisplatin in the target and bystander groups in comparison with the control group showed a 6.003 and 1.182 fold increase, respectively. In addition, the expression of P53 in the target and bystander groups in US exposure with cisplatin had 8.208-and-1.373 fold increases (P<0.001).

Figure 6 showed reduced HO-1 expression in US exposure without cisplatin in the target and bystander groups (P<0.05). The level of HO-1 gene expression in these groups had 0.62 and 0.8 fold decreases, also in US exposure

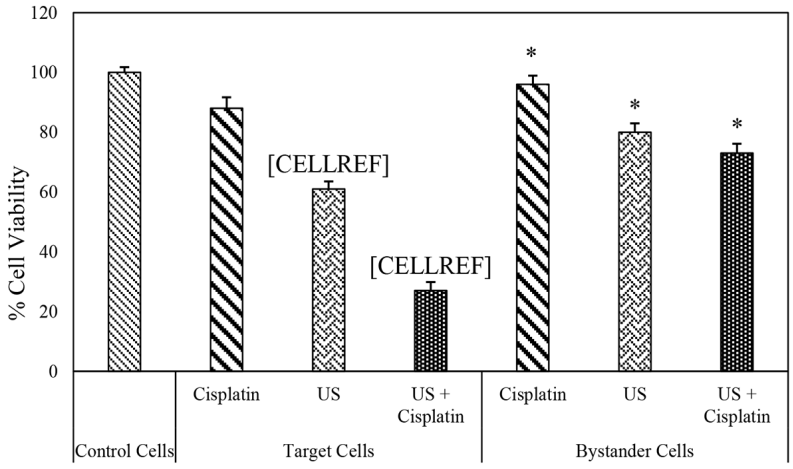

Figure 3: Cell viability in target and bystander cells with or without cisplatin treatment after US exposure. Error bars show standard deviation (SD). Data are expressed as mean±SD (n=3) in comparison with the control group. *Significant difference in the comparison with the control group (*P<0.05, **P<0.01, ***P<0.001).

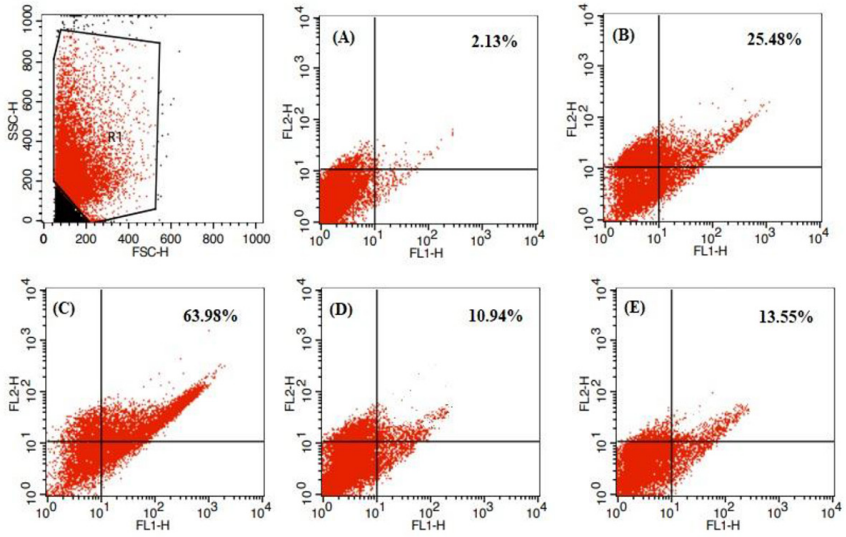

Figure 4: Contour diagram of Annexin V/PI (Propidium Iodide) flow cytometry of A375 cells after treatments. Unexposed cells were used as a control (A). US (Ultrasound) exposure A375 cells without (B) and with (C) cisplatin treatment in target cells, US exposure without (D) and with (E) cisplatin treatment in bystander cells.

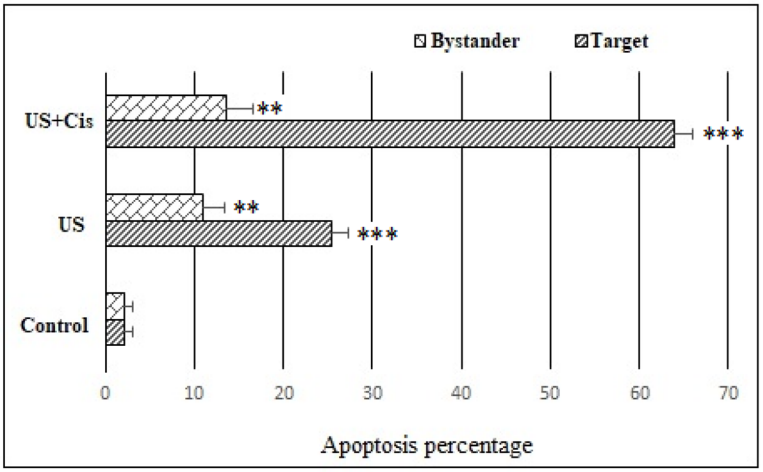

Figure 5: Percentage of apoptosis and necrosis in bystander groups. Error bars show standard deviation (SD). Data are expressed as mean±SD (n=3) in comparison with the control group. *Significant difference in comparison with the control group (*P<0.05, **P<0.01, ***P<0.001).

with cisplatin gene expression in the target and bystander groups had 2.69- and 2.03-fold increases, respectively (P<0.001). Increasing gene expression in the target cells is higher than those of the bystander cells (P<0.001).

Discussion

The purpose of this study was to determine the bystander effect of 1 MHz therapeutic US exposure with or without cisplatin on the human melanoma cell line. Figure 3 shows that the US waves cause the target cell's death and the percentage of cell death in the US irradiation in the presence of cisplatin is higher than the US irradiation alone. Our findings are consistent with the Bernard et al. study that

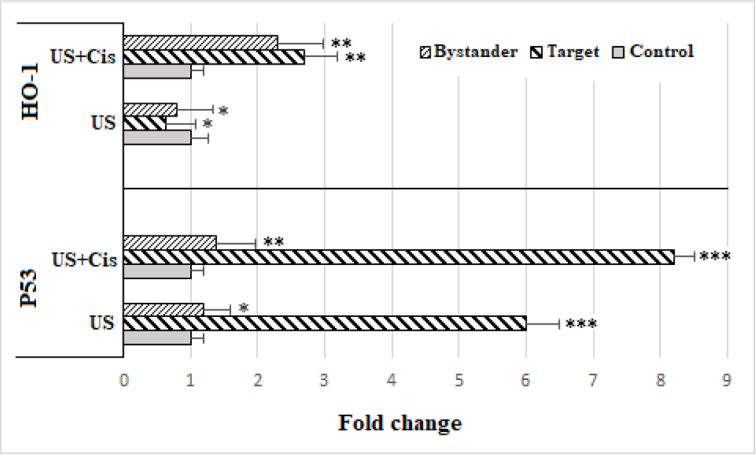

Figure 6: The expression levels of P53 and HO-1 genes in the target and bystander groups. Error bars show standard deviation (SD). Data are expressed as mean±SD (n=3) in comparison with the control group. *Significant difference in the comparison with the control group (*P<0.05, **P<0.01, ***P<0.001).

examined the effect of US exposure alone and in combination with cisplatin on the A375 cell line [19].

According to Figure 3 in bystander groups, a statistical comparison of the results shows a significant difference in cell viability between bystander groups receiving cell culture medium in the target groups irradiated with or without cisplatin control group after 24 h of incubation, i.e., therapeutic US waves can induce cell death in the bystander cells. In other words, the target cell culture medium irradiated with ultrasound contains signals leading to cell death in bystander cells.

The most remarkable result that emerged from the flow cytometry was that the culture media of target cells irradiated with the US could induce and enhance apoptosis in bystander cells by cisplatin.

In the classical approach, US irradiation on target cells demonstrates increased apoptotic cells [22], although to the best of our knowledge no study has investigated this effect in bystander cells. The present study examined transcription changes in the bystander cells using the real-time polymerase chain reaction and focused on the expression of P53 and HO-1 genes for the investigation of the molecular aspect of induced cell death by bystander effect.

One of the genes that sense DNA damage is P53, contributing to DNA repair, cell cycle regulation, and apoptosis. In addition, the most critical effect of P53 is arresting damaged cells in the G1/S phase to extend the G1 phase, resulting in increasing the cell chance to repair their DNA damages and preventing the transmission of the damaged gene to daughter cells [23]. However, the level of P53 gene expression increased significantly in the target and bystander cells irradiated with the US with or without cisplatin compared to the control group.

In a previous study, Rezaei et al. investigated the bystander effect due to US radiation in A375 and the level of P53 gene expression increased significantly in the target and bystander cells irradiated with the US compared to the control group. The results of the current study are consistent with Rezaei et al. [10] findings. Also, the results of a study by Bohari et al. represented an increasing level of P53 gene expression for directly exposed MCF-7 cells after US irradiation [24].

Olsson et al. in their experiments with ionizing radiation on the HepG2 cell line observed

increased P53 gene expression levels in the bystander cells [25], i.e. non-ionizing waves can also cause bystander effects and ionizing waves.

In another study, Koturbash et al. investigated the bystander effects in a mouse model by irradiating a part of the scalp of a mouse with ionizing radiation while covering the rest of the body with lead shields [23] and their results showed a significant increase in P53 gene expression in spleen cells as the bystander tissue;

HO-1 is another gene evaluated in this study. HO-1 is an antioxidant enzyme exhibiting significant anti-inflammatory and anti-apoptotic functions [26-29]. Was et al. exhibited that HO-1 gene overexpression increases tumor cell proliferation and improves angiogenic capability [30]. HO-1 causes cell resistance against oxidative stress, leading to melanoma cancer's aggressiveness and metastatic nature both in vitro and in vivo [30]. Thus, the down-regulation of HO-1 might be beneficial in melanoma therapy and increase apoptosis in melanoma cells.

As shown in Figure 6, in this study, US waves without cisplatin in the target and bystander cells inhibited the expression of the HO-1 gene.

In the present study, an increase in the apoptotic of bystander cells agrees with changes in the expression of P53 and HO-1 genes. The apoptosis in bystander cells may correlate with increasing P53 expression as a promoter of apoptosis and decreasing HO-1 expression as an apoptosis inhibitor.

However, the expression of the HO-1 gene in target and bystander cells with cisplatin showed an increase that is inconsistent with the results of the studies mentioned earlier. In other words, decreasing the HO-1 gene expression resulting from the bystander effect is a favorable effect and inhibits tumor cell growth.

According to the finding of the present study, low-intensity US waves alone and in combination with cisplatin could create bystander signals in the culture medium of target cells and induce apoptosis and changes in P53 and HO-1 gene expression in target and bystander cells. Further studies are suggested to determine protein level changes.

Conclusion

The present study describes the effect of the combinational therapeutic US and cisplatin on inducing bystander effect in melanoma. Based on the results, the US waves with or without cisplatin can create bystander signals in the culture medium of target cells, resulting in inducing apoptosis and changes in P53 and HO-1 gene expression in non-treated cells. Therefore, US waves similar to ionizing radiation can induce the bystander effect and the presence of cisplatin can increase this effect.

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Authors' Contribution

M. Rezaei performed in-vitro experiments, A. Esmailzadeh performed data analysis, drafted the manuscript, and edited it, and A. Shanei supervised the overall study design and provided scientific advice. All the authors read, modified, and approved the final version of the manuscript.

Ethical Approval

Not applicable, because this article does not contain any studies with human or animal.

Conflict of Interest

None

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