Enhanced antitumor effects of low-frequency ultrasound combined with adriamycin on human leukemia multidrug resistance cell line K562/A02

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Background and Objective: Research has shown that ultrasound can enhance the sensitivity of tumor cells to chemotherapy drugs and therefore inhibit cell proliferation. This study investigates the antitumor effect of low-frequency ultrasound combined with adriamycin on human leukemia multidrug resistance (MDR) cell line K562/A02. Methods: K562/A02 cells were divided into four groups: blank control group, adriamycin group, ultrasound group and adriamycin plus ultrasound group. The trypan blue dye exclusion assay and MTT assay were used to determine the viability and proliferation of K562/A02 cells, while Wright's stain and flow cytometry were used to determine the apoptosis and the concentration of adriamycin. The expression of P-glycoproteins (P-gp) was detected using immunohistochemistry. Results: Ultrasound (20 kHz, 0.25 W/cm², 60 s) combined with adriamycin (7.5 μg/mL) induced more apoptosis of K562/A02 cells than adriamycin alone. Compared with the adriamycin group, ultrasound at a frequency of 20 kHz and an intensity of 0.5 W/cm² exerted an acute killing effect on cells. Ultrasound increased the intracellular concentration of adriamycin and promoted apoptosis of K562/A02 cells but did not change the expression of P-gp on the cell membrane. Conclusion: Ultrasound at a frequency of 20 kHz, an intensity of 0.25 W/cm² and duration of 60 s can enhance the killing effect of adriamycin on K562/A02 cells.

Given the existence of multidrug resistance in tumors, the treatment outcome for hematological cancers is not satisfying. In order to improve the efficacy of chemotherapy drugs, supplemental physical therapies are often used concurrently. In recent years, ultrasound treatment of tumors has attracted increasing attention. Among One, high-intensity focused ultrasound (HIFU) takes advantage of many ultrasound effects such as the infiltration-promoting effect, warm-generating and cavitation effect to enhance the cells’ uptake ability, and the toxic effect of P-glycoprotein (P-gp) to increase the toxicity of lipophilic anti-cancer drugs. HIFU works mainly through its warming effect, which can not be easily controlled. Recently, low-energy ultrasound treatment of tumors has become a popular subject in oncology. Therefore, in this study we focused on screening the proper ultrasound intervention parameters on drug resistant leukemia cells in order to provide guidance for in vitro cell purification treatment.

Materials and Methods

Materials. Cells. Human red blood cell leukemia multidrug resistant cell line K562/A02 was provided by the Chinese Academy of Medical Sciences Institute of Hematology, cultured in RPMI-1640 containing 10% neonate calf serum, 100 u/mL penicillin and 100 μg/mL streptomycin, at 37°C and 5% CO₂.

Equipment. NTY3000 utility ultrasonic surgical device (the NTY device) was produced by the MEIDAKANG Medical Equipment Factory of Southeast University, with a power output of 0–100 W, frequency of 20.24 kHz.

Experimental methods. Experimental groups. Cells in logarithmic growth phase were diluted into 5 x 10³ suspension solution and placed into 24-well plates at 1.0 mL per well. Samples were divided into four groups: the blank control group did not receive adriamycin; the adriamycin group respectively received 0, 0.5, 1.0, 2.0, 3.0, 5.0, 7.5, 10.0, 15.0, 20.0, 25.0 and 30.0 μg/mL adriamycin in order to test the proper adriamycin concentration in this study; the ultrasound only group received ultrasound intervention of different intensities (0.1, 0.17, 0.25 and 0.50 W/cm²) and time (30, 60 and 120 s), in order to screen the proper intervention parameters for ultrasound treatment; the ultrasound...
+ adriamycin group received adriamycin followed by ultrasonic radiation (frequency of 20 kHz, irradiation time of 60 s, sound intensity of 0, 0.1, 0.17 and 0.25 W/cm²).

Trypan blue staining assay to count living cells. K562/A02 cells of 5.0 x 10⁵/mL were placed in 24-well plate (1.0 mL/well) and routinely cultured, three parallel wells per group. Cells were collected after being radiated for respectively 24 and 48 h, and an equal volume of 0.2% trypan blue dye was added and counted on a hemacytometer under light microscope. Cells stained with blue color were dead cells and survival cells were counted. Each sample was counted twice and 100 cells were counted each time. The percentage of living cells = living cells/(number of living cells + dead cells) x 100%. Experiments were repeated three times and mean plus standard deviation was calculated.

MTT assay to test cell proliferation. After intervention cells were changed into ice cold culture medium and plated into a 96-well plate, 100 μL per well at 2 x 10⁴; three duplicate wells were plated for each group and cultured in incubator. MTT solution (10 μL at 5 μg/mL) was added into each well respectively at six, 24 and 48 h, stored at 37°C for 4 h, and centrifuged at 500 xg for ten min, the supernatant was discarded and 100 μL dimethyl sulfoxide (DMSO) was added into each well. After total dissolving of precipitations, absorption value (A value) was read on a spectrometer. The cell proliferation rate was calculated according to the following equation:

\[ \text{Proliferation rate} = (1 - \text{A value of experimental well}/\text{A value of control well}) \times 100\% \]

Flow cytometry to measure intracellular adriamycin concentration. After being incubated at 37°C for six and 24 h following the intervention, cells were washed by pre-cooled PBS, separated by centrifuge, and washed and centrifuged again. Fluorescence intensity of intracellular adriamycin was measured by flow cytometry.

Detection of apoptosis. Cells were collected for centrifuge 6 h and 24 h after irradiation, spread on a slide and fixed by methanol; specimens were stained by Wright-Giemsa's compound for 20 min after drying and were observed under light microscope.

Detection of P-gp. After intervention, absorbed cells were put on slides and operation was performed following manual instructions. Specifically, cells were plated in different polyethylene tubes and treated respectively according to grouping. Film slides of cell suspension were blow-dried and fixed for 2 min. PBS was used to wash away floating cells and specific immunohistochemistry was performed according to reagent instruction. Film slides were labeled by anti-P-gp monoclonal first antibody and horseradish peroxidase tagged secondary antibody, luminescence-developed, washed by PBS three times, mounted and observed under the microscope. The criteria for MDR1-Ab negative staining was that no positive staining substances were on the cell membrane; if positive staining substances appeared on cell membrane or inside cytoplasm (brown-yellow or brown-red color), it was considered as positive; if positive staining substances appeared as brown-black color and were distributed in sheet-shape, it was considered as a strong positive. Normal sheep serum instead of antibody (MDR1-Ab) blocked slides were used as negative control.

Statistical analysis. SPSS12.0 software was used for statistical analysis. Analysis of variance and t test were performed using the software and p < 0.05 was set as the criteria for statistical difference. SPSS11.0 software was used to calculate IC₅₀ and JIN’s formula was applied to determine the outcome of combined intervention:

\[ Q = \frac{E_a + b}{E_a + Eb - E_a \times Eb} \]

\[ E_a + b \] represents cell inhibition rate of combined intervention; \( E_a, Eb \) represents the cell inhibition rate of A and B intervention alone; the numerator represents the actual combined effect while the denominator represents the expected combined effect, \( Q \) represents the ratio of the two values. \( Q \) value of 0.85 to 1.15 indicated a simple addition, and 0.50 to 0.85 an obvious enhanced effect, >2.0 an obviously enhanced effect, 0.85 to 0.55 an antagonistic effect and 0.55 an obviously antagonistic one.

Results

Experiment concentration of adriamycin. The half inhibitory concentration (IC₅₀) of adriamycin on K562/A02 was 22.30 μg/mL and the experimental toxic range of adriamycin on K562/A02 cell lines was 0–22.30 μg/mL.

The effect of simple ultrasound of different intensity and radiation time on tumor cells. After one h of ultrasonic radiation, the cell survival rate decreased with the increase of ultrasound dose. 0.50 W/cm² dose of ultrasound produced acute killing effect while ultrasound within 120 s and intensity below 0.25 W/cm² did not affect the survival time of K562/A02 cells significantly (p > 0.05) (Fig. 1).

The effect on cell survival rate of ultrasound combined with adriamycin. Along with the increase of ultrasound intensity, the same concentration of adriamycin had a significantly different inhibitory effect on cells than the drug alone (p < 0.05) (Fig. 2).

The IC₅₀ value of different ultrasound parameters. The IC₅₀ of adriamycin alone on K562/A02 cells was 22.30 μg/mL. Under 0.25 W/cm² ultrasound concentration the IC₅₀ of adriamycin concentration was 7.5 μg/mL; under 0.17 W/cm² ultrasound the IC₅₀ of adriamycin concentration was 12.5 μg/mL; under 0.1 W/cm² ultrasound the IC₅₀ of adriamycin concentration was 17.5 μg/mL.

Intracellular concentration of adriamycin. Ultrasound can promote the adriamycin uptake of cells. The intracellular adriamycin fluorescence intensity in blank control, 7.5 μg/mL adriamycin group and ultrasound (0.25 W/cm², 60 s) + adriamycin intervention group were respectively 2.31, 20.89 and 23.46 at six h, and 2.93, 48.04 and 55.28 at 24 h.

Detection of apoptosis. Obvious cells apoptosis appeared in cells treated by ultrasound and adriamycin (0.25 W/cm² US + 7.5 μg/mL adriamycin) The apoptosis rate after different interventions for six or 24 h were respectively, blank control group 0.98% and 1.38%; adriamycin alone 12.24% and 20.20%; simple ultrasound group 9.05% and 16.87%; adriamycin and ultrasound joint group: 27.82% and 46.90%. Q values of the above intervention were 1.338, 1.45 (>1.15), indicating that ultrasound radiation combined with adriamycin had synergistic effects, killing K562/A02 cells.

Expression of P-gp after ultrasonic radiation. No significant difference was detected between the K562/A02 cells P-gp positive rate before and after the ultrasound radiation (p > 0.05).
Discussion

The multidrug-resistance phenomenon (MDR) of tumor cells manifests as the enhancement of anti-apoptosis ability, irrational cell proliferation and inhibition of apoptosis, which is the major cause of tumor occurrence. Defects in apoptosis induction lead to uncontrollable tumor cell growth and are favorable for the formation of tumors. Therefore apoptosis resistance is the foundation for tumor formation. Chemotherapy mainly functions through inducing tumor cell apoptosis, since inducing tumor cell apoptosis will inhibit the growth of tumor cells. In recent years, a large number of experimental and clinical studies have shown the great potential of low-frequency ultrasound in clinical application. As a new technology, ultrasound has been used in the clinical treatment of tumors.

Low-frequency ultrasound primarily inhibits cell proliferation through its heating, cavitation and mechanical effect. Research shows that the biological effectiveness of ultrasound is negatively correlated with ultrasound frequency; in this study, we selected a lower ultrasound frequency of 20.24 kHz. Pan C, et al. believe that simple low-frequency (20 kHz, 0.2 W/cm²) ultrasound continuously radiating Molt4 tumor cells for one min significantly induces cell apoptosis and inhibits tumor cell proliferation. Our study found no significant difference after 20.4 kHz, less than 0.25 W/cm² ultrasonic radiated for 60 s, while stronger intensity led to acute cell death. This may relate to individual differences in tumor cells. Lagneaux et al. find that the “blood purification” therapy for treatment of hematologic tumors can be achieved by inhibiting tumor cells by low-energy ultrasound. In addition, the apoptosis induction time and dose of low-energy ultrasound on different tumor cells such as HL60, K562, KG1a and Nalm6 are different, so individualized ultrasound therapy should be introduced on a clinical level.

Intracellular adriamycin concentration detected by flow cytometry in this study showed that the amount of adriamycin entering cells after ultrasound irradiation increased significantly, consistent with the belief of some researchers that raising temperature can increase the drug concentration within cells. However, according to empirical formula, the selected ultrasound parameters in this study had no effect on temperature. Therefore, we believe that the low-power low-frequency ultrasound in this study mainly affects cells through cavitation; generation of cavitation nucleus and its breakdown is the major reason for low-frequency ultrasound to promote penetration. The greater the intensity, the higher the concentration the cavitation nucleus will be and the more significant the cavitation effects will be. Cavitation can produce high-temperature and high pressure, as well as generate shock wave phenomena, high-speed jet and free radicals. Adjacent cells under the above-mentioned phenomenon form “sound hole” channels, which open to surrounding large molecules and is called “sound hole effect.” When used together with chemotherapy drugs, low-intensity ultrasound can change cell membrane structure so that drugs enter the cell more easily, therefore enhancing its toxicity. Ultrasound irradiation combined with adriamycin significantly increased its efficacy and reduced the IC₅₀ value of adriamycin, indicating that ultrasonic radiation of such parameters can significantly increase adriamycin concentration to strengthen the cells’ killing effect while simple ultrasound alone had little effect on cells.

The morphological changes that appear in the apoptosis process present as chromatin condensation, aggregation and formation of nuclear debris, and eventually the formation of apoptotic bodies, which is the major characteristic of apoptotic cells. In this study, ultrasound of chosen parameters combined with adriamycin increased cell apoptosis of typical morphological changes. Q values at six and 24 h were all greater than 1.15, indicating that ultrasonic radiation and adriamycin had a synergistic effect on K562/A02 cells.

Among the drug resistant mechanisms of cells, P-gp plays an important role. In this study, K562/A02 cell line also had high expression of P-gp. However, no significant changes were identified in its expression level after ultrasonic radiation, suggesting that low-frequency low-intensity ultrasound radiation does not affect P-gp expression, consistent with other reports.

Tumor cells are more sensitive to ultrasound compared with normal cells and there is a “threshold” dose which effectively...
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treats tumor cells with little effect on normal cell. This theory provides a foundation for clinical application of ultrasound. In this study, our result provides certain guidance for blood purification therapy. However, the subjects of in vitro study differ from in vivo treatment, which need to be considered in clinical applications and further studied. In addition, the mechanisms by which low-frequency low-intensity ultrasound reverses tumor drug resistance still need to be further investigated.

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References