

Comparing the Effect of Physical Modalities on Permeabilisation of Cells to Bleomycin in Balb/C Mice

Bahram Yousefian,¹ S. Mohammad Firoozabadi,^{*1} Manijeh Mokhtari-Dizaji¹

1. Department of Medical Physics, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran

Article information	Abstract
<p>Article history: Received: 4 July 2010 Accepted: 10 Apr 2011 Available online: 13 Feb 2012</p> <p>Keywords: Permeability Bleomycin Magnetic field Balb/c</p> <p>*Corresponding author at: Department of Medical Physics, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran. E-mail: pourmir@modares.ac.ir</p>	<p>Background: Some physical factors may facilitate the entry of chemotherapeutic drugs to the cells. In this study, permeability level of tumor cells of murine breast adenocarcinoma to Bleomycin was compared with 5 minutes ultrasonic exposure vs. magnetic field of 3.5 Tesla in Balb/c mice.</p> <p>Materials and Methods: In this experimental-applied study, 80 five-week female Balb/c mice were purchased from Pasteur Institute of Tehran. After 10 days, skin tumors of mice were induced through Homograft, and they were randomly classified after tumor reached a treatable size. In ultrasound combination group, intratumoral injection of bleomycin was performed on anesthetized mice and three minutes later, the mice, which were placed in the sonication chamber, were put in a water tank in the exposure position, and the tumor was exposed to ultrasound for 5 minutes. In the magnetic field group, mice were placed in a handmade chamber after intratumoral injection of bleomycin. Three minutes after injection of bleomycin, eight pulses of 3.5 Tesla magnetic fields with 1_{Hz} frequency were applied to each one of the tumors.</p> <p>Results: It yield that, eight 3.5 Tesla pulses of magnetic field, was slightly more effective than 5 min ultrasonic irradiation in cells permeability to bleomycin, but these two physical factors had no statistically significant difference.</p> <p>Conclusion: Tests showed that these two physical factors have similar effects and use of each depends on the position of the patient and the medical center's facilities.</p> <p>Copyright © 2012 Zahedan University of Medical Sciences. All rights reserved.</p>

Introduction

Breast cancer is the second most common fatal cancer in women. Breast cancer includes 22.9 percent of all cancers (except non-melanoma skin cancer) around the world. According to the America Cancer Society, annually approximately 1,300,000 women around the world are diagnosed with this disease and about 465,000 of whom die of this disease. In the United States, one out of every 35 women dies of breast cancer. In the United Kingdom, annually 45,000 cases are diagnosed with breast cancer 12,500 of whom die of this disease. In South America in 2001, the number of new cases and expected deaths are respectively 70,000 and 30,000 [1].

In cases where surgery is not performable, combined treatment is used in order to reduce damages to healthy tissue during chemotherapy and enhance the efficiency of the treatment. These treatments result from the combination of drug and a certain physical factor. Physical factors, which have been so far used for this purpose, are including ultrasound, laser, electric field and recently magnetic field [2-7]. The use of physical factors along with drugs have at least two clear advantages: First, the dose of the used drug will be reduced, second, since physical factors affects only the tumor, the healthy tissue will be more preserved. The competition between these physical factors was also interesting. Among all of them,

two factors, namely ultrasound and electric field, which proved their more efficiency, were compared in some paper [8-9]. In one of these studies, in order to evaluate the therapeutic potential provided by ultrasonic irradiation vs. electric field exposure, the antitumor effects of combined chemotherapy with these two physical modalities investigated and the efficiency of these two methods in gene transfer has been compared. Thus, the ability of both physical factors in cell permeability to bleomycin is the same, but gene transfer by electroporation has shown a greater productivity. Although the magnetic field easily passes through the body ($\mu=1$), but it is proven not to have effect on the body, therefore field must be time-varying so that it could induce an electric field in the body depth according to Maxwell's third law. In fact, this induced electric field, which is of very low intensity, does the permeability.

Recently, researchers have revealed that low-intensity electric fields (2.5-20 V/cm) are able to attract and harvest macromolecules by cells [10-11].

In addition, using computer simulations in another study, it has been revealed that pulsed magnetic field of 3.5 Tesla is able to build induced electric field of 7.5 V/cm at a distance of one cm from the probe [12]. Unlike ultrasound, magnetic field does not need transitional environment to enter the body tissues and unlike electric

field, different layers of skin and other body tissues do not have any resistance against magnetic field ($\mu=1$). Thus, in this study, in order to measure the ability of magnetic field, which is a new and completely non-invasive method for cell Permeability, the effect of low-intensity ultrasound ($2\text{W}/\text{cm}^2$) with irradiation time of 5 minutes and 80% duty factor on Permeability of murine breast adenocarcinoma tumor cells to anticancer bleomycin drug has been compared with apply of eight pulses of magnetic field of 3.5 Tesla in this respect. Pulsed magnetic field has not been so far applied without using magnetic nanoparticles and for Permeability of tumor cells.

Materials and Methods

In this experimental-applied study, 80 five-week healthy female Balb/C mice were gradually and in a few stages purchased from Pasteur Institute (Tehran-Iran). In order to adapt to the new environment and eliminate the potential stress, animals were kept under standard laboratory conditions for 10 days before tumor induction.

64mm^3 pieces of murine Breast Adenocarcinoma tumor were induced under the skin of right flank of mice through Homograft, and they were randomly classified after tumor reached a treatable size. The induction of tumors to mice and monitoring the tumor size is exactly the same as the methods employed in related articles. The standard approach that the other researchers have followed is also used in exposure methods [20, 22, 23].

Selection of the number of samples is based on the sample number in works similar to other physical factors. This research has been conducted in 2010 at Tarbiat Modares University.

Ultrasonic exposure setup: the ultrasound device used is made by the Dutch Enraf Nonius Company, Sono Puls 492 Model and with a PZT transducer (Diameter 30 mm and an area 5cm^2) with continuous and pulsed operation modes. Audio calibration of this device was performed in terms of power and intensity using a balance ultrasound power meter (UPM-DT-10, Netech, Hicksville, NY, USA, ± 1 mW) in dissolved gas free water. The tested intensity was measured based on spatial average, time average (I_{SATA}) and for $2\text{W}/\text{cm}^2$.

For sonication under controlled conditions, a cube-shaped chamber was made of Plexiglas with dimensions of $20 \times 25 \times 25$ cm. At a distance of 1cm from one of the vertical dimensions of the chamber, a circular duct was made in which the ultrasonic probe was workout and sealed using aquarium silicone sealant. To avoid air bubbles between the probe and the tumor, a small amount of liquid detergent was added to the tank water. In order to perform tests under progressive wave conditions and limit the sound reflection from the tank wall, a piece of an ultrasound absorbent material was attached on the internal surface of the wall which was in front of the probe in the water tank.

To maintain water temperatures between $35\text{-}38^\circ\text{C}$ during testing, an Aquarium Heater with adjustable thermostat was used. Also during the performance of all tests, water

temperature was also continuously measured by a digital thermometer.

To keep the tumors of the mice in contact with the probe, a small chamber containing three rings connected by Plexiglas wires was used. The diameter of wires was different and the three of them were thick and the rest were thin and at proper distance for exposure. Chamber system had the ability to move in three dimensions. Thus, it was quite possible to put the tumor in contact with the probe at exposure position. A view of the ultrasonic exposure setup is shown in figure 1.

Magnetic field application tools: a nerve stimulator device brand MAGSTIM (MAGSTIM[®] Rapid UK Pat. No. GB2298370B) was used to produce the desired magnetic field. This device is normally used to treat epileptic patients. Since the maximum intensity of field produced by the device should have been used, the probe with the smallest diameter should have been used and this required the use of an additional inductor in the connection route of the probe to the device.

A handmade chamber was used to expose mice to the magnetic field. Mice with tumors were placed in the chamber and the chamber was put one centimeter from the probe that was placed on a flat stable surface. Eight pulses of magnetic field the intensity of each was 3.5 Tesla and duration of each was $160\ \mu\text{s}$ at the frequency of 1Hz were applied to any of the mice that were needed.

Tumor induction: murine adenocarcinoma tumor was extracted from the stock mouse after the anesthesia. Tumor in PBS solution Containing 2% Penesterpt was divided into cube-shaped pieces with approximately 4mm length of each crest. The tumor was induced under the skin of the right flank of mice through Homograft. 2-3 weeks after Homograft, tumors were reached the treatable size ($250\text{-}500\ \text{mm}^3$).

The treatment was conducted on mice according to their experimental groups [13, 14]. Drug Injection: the potency of bleomycin is measured in units of antimicrobial activity. One unit (U) contains 0.56-0.66 mg of bleomycin and each unit is equivalent to 1000 international units ($1\text{U}=1000\ \text{IU}$) [15, 16] Bleomycin is used as an anti neoplastic agent in the treatment of Hodgkin's disease, non-Hodgkin lymphomas and testicular cancer [14].

One ml of saline injected to each bleomycin vial which contained 15 mg Bleomycin in the form of powder. Therefore, each vial in this case contains 25U drug and therefore there was 2.5 unit drugs in each 0.1ml of it. Considering the tumor size, the appropriate dose of drug was injected directly into the tumor. The injection was performed in two opposite points of tumor to help the uniform distribution of drug among tumor cells. The dependency of the injected dose of drug on tumor size is summarized in table 1.

Anesthesia: Anesthesia solution consisted of a combination of 4 ml injectable saline and 0.5 ml 10% ketamine (Alfasan Woerden-Holland) and 0.5 ml of xylazine 2% (Alfasan Woerden-Holland). For each gram of mouse weight, 0.01 ml of anesthetic solution was intraperitoneally injected by use of an insulin syringe.

Ultrasonic exposure: if necessary, the anesthetized mouse was injected with the appropriate dose of bleomycin and then was put into the exposure chamber. Three minutes after injection of bleomycin, the chamber containing mouse was put in the water tank in front of the probe so that the tumor would be exactly close and against the probe. The tumor will be exposed to ultrasound with intensity of $2\text{W}/\text{cm}^2$ and duty factor 80% at the frequency of 1MHz for 5 minutes.

Magnetic exposure: In the selected groups that bleomycin injection was essential, after injection of the proper dose of bleomycin in the two opposite points of the tumor, mice were placed in the exposure chambers and 3 minutes after the injection, the magnetic field was applied. Eight pulses of 3.5 Tesla Magnetic field at the frequency of 1Hz and 160 μs pulse duration was used.

Experimental groups: There were eight experimental groups in this study as follows: 1 - Sham control group (Sham Cont.): 0.1 ml of distilled water was injected into the two opposite points of the tumor. 2 - Bleomycin control group (BLM Cont.): 0.1 ml solution of bleomycin was injected into two opposite points of the tumor. No radiation was applied. 3 - Bleomycin plus 3.5 Tesla magnetic field group BLM plus 3.5 Tesla MF: After injection of the appropriate dose of bleomycin into the opposite points of the tumor, mice were placed in the exposure chamber and three minutes after the injection, the magnetic field was applied through the described method. 4 - Sham magnetic field group (Sham MF): treatable size tumors were placed in contact with a magnetic stimulator probe for 8 seconds. No drug or distilled water was used. No field was applied. 5 - Only magnetic field group (Only MF): treatable size tumor mice were placed in contact with a magnetic stimulator probe. Eight magnetic field pulses were applied through the described method. No drug or distilled water was used. 6 - Bleomycin plus 5 minutes ultrasound group (BLM plus 5min US): after anesthesia in mice, 0.1 ml bleomycin was injected into the two opposite points of tumor, and then the animal was put in the exposure chamber and set it in front of the probe into the water and ultrasound exposure starts three minutes after bleomycin injection for 5min. 7 - Sham ultrasound(Sham US): except for the drug injection and exposure, all the processes performed for the previous group, are also done in this group. 8 - Only ultrasound group (Only US): It is similar to the sham ultrasound group, with the difference that ultrasound exposure is done in this group.

Monitoring tumor: tumor diameter was measured once every 48 hours using a digital caliper with an accuracy of 0.01 mm. Before any measurements, hair around and on the tumor was removed using Hair Removal Cream (epilating cream) if needed to provide the measurement accuracy. Then, tumor size was calculated using the standard formula. The formula most often used to measure the tumor volume is $V = \frac{ab^2}{6}$ in which (a) is the longest diameter and (b) the next longest diameter perpendicular to (a) [13].

Tumor growth graph was extracted and plotted in different experimental groups by use of Excel software. Using computer software SPSS-18, statistical analysis of data was performed through ANOVA.

Table 1. Dependence of bleomycin dose on tumor size

No	Volume of tumor (mm ³)	Drug dose (U*)	BLM (mg/0.1ml)
1	< 100	0.5	0.3025
2	100-150	0.75	0.45375
3	150-200	1	0.605
4	200-250	1.5	0.9075
5	250-300	2	1.21
6	>300	2.5	1.5125

*One unit (U) contains 0.56-0.66 mg of bleomycin



Figure 1. View of the ultrasonic exposure setup containing rats

Results

Tumor growth graph in different experimental groups is shown in figure 1. Data analysis by use of statistical software SPSS-18 at confidence level of 0.05 showed no significant difference in cell Permeability between the two treatment groups of BLM plus 3.5 Tesla MF vs. BLM plus 5min US. This fact is evident by looking at the tumor growth graph.

There was a significant difference between the Sham Control. Group vs. BLM plus 3.5 Tesla MF at the 18th, 20 and 22nd and vs. Only US group at 22nd days after treatment. BLM Cont. group at 14th, 16th, 18th and 22nd days has significant difference vs. Only US group. It also gets significant difference vs. BLM plus 3.5 Tesla MF at 18th and 20th days. BLM plus 5min US at the 10th, 12th, 14th, 16th, 18th, 20th and 22nd days showed significant difference vs. only US group. It also has significant difference vs. Sham US and sham MF groups at 14th, 18th, 20th and 22nd days ($p < 0.05$). BLM plus 3.5 Tesla MF showed significant difference vs. Only MF group at 4th day. It also has significant difference vs. Sham US and Only US groups at 6th, 8th, 10th, 12th, 14th, 16th, 18th, 20th and 22nd days ($p < 0.05$). It must be mentioned that this group has also significant difference vs. Sham MF group at 10th, 12th, 14th, 16th, 18th, 20th and 22nd days ($p < 0.05$; SEM=1.0072E3).

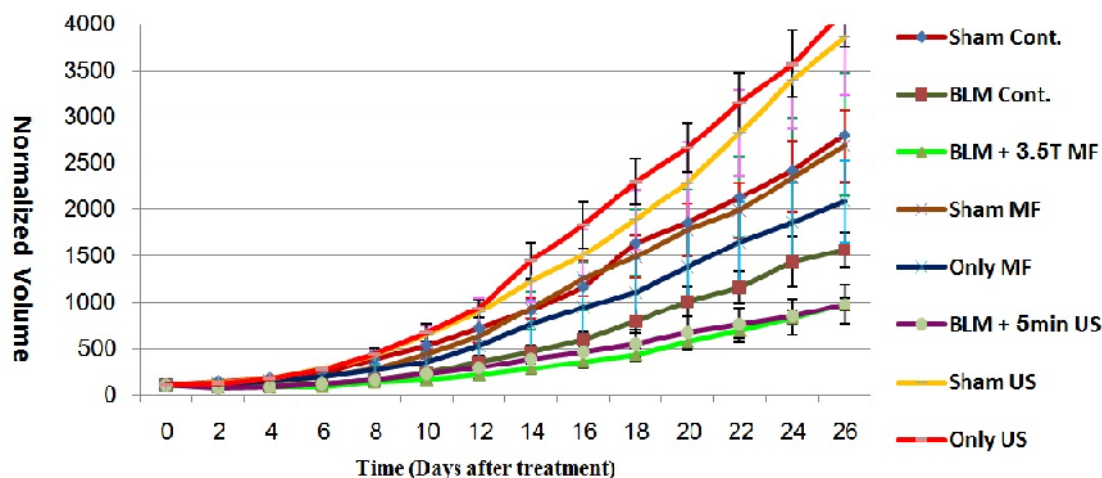


Figure 1. Tumor growth curve in different experimental groups of 8 different groups of Balb/C mice (n=10) with breast adenocarcinoma tumors. Sham control, □; bleomycin control, ○; Bleomycin + 3.5 Tesla magnetic field, △; sham magnetic field, ×; only magnetic field, *; Bleomycin + 5 min ultrasound, ◇; sham ultrasound, +; only ultrasound, □; Standard error Markers are also shown.

Discussion

Lack of significant difference between BLM Cont. group and combined treatment groups showed the minor effects of combining drugs and physical agents to output a synergism effect on tumor control. Thus, it seems that the application of physical factors in this study has not been enough to create the significant effect on permeability of tumor cells to drug and turnout a synergism effect on tumor growth control.

There was a highly significant difference between BLM Control. vs. sham US and only US (p -Value are 0.014, 0.004 respectively). Although, there were no significant difference between it vs. Sham Cont. and Sham MF and Only MF. The reason of this difference can be surveyed from two aspects. First, the method of performing test in ultrasound and magnetic field groups - that in ultrasound animal is being tested while anesthetized- and therefore it felt no stress. While in the magnetic field groups, there was a completely great stress during the test due to the lack of anesthesia.

This stress may also reduce tumor growth in magnetic field groups and make their results similar to the BLM Controls. The second reason lies in the difference between nature of ultrasound and magnetic field. Low-intensity ultrasound and the terms used in this study were not able to create a transient cavitation phenomenon, and if cavitation has been occurred, it has been necessarily inertial cavitation [17, 19]. Therefore, ultimately all ultrasonic energy will turn into heat and will increase the ambient temperature. Rising temperatures will also cause vasodilatation and increase tumor perfusion, which will lead to better nutrition and more oxygen for tumor and its increasing growth. While the magnetic field does not have this effect and the studies have shown that exposure to electric fields can also cause vasoconstriction [19, 20]. Although the induced electric field caused by the variable magnetic field used in this test is low-intensity, it has not been ineffective. Thus, we realize that the obtained results

are quite consistent with these facts. Despite the fact that BLM plus 3.5 Tesla MF group shows a significant difference with all sham groups as well as only US group, it has no significant difference with only MF group. This fact once again shows the inhibiting effect of the magnetic field on tumor growth.

Lack of significant difference between sham MF group vs. BLM plus 5min US group is also interesting. The difference between sham MF group vs. BLM and 3.5 Tesla MF group is also weakly significant. In addition to the stress during test performance, the pulse generator and nerve-stimulating device in passive mode may also generate electric fields around themselves; electric fields with deterrent effect on tumor growth. Further research is necessary in this regard.

Another interesting issue is that the only MF group shows a significant difference with only US group. This phenomenon again confirms the validity of our conclusions about the inhibiting effect of tumor growth for the magnetic field and even though ultrasound is not carcinogenic, but it confirms the facilitating effect on the tumor cells growth at low-intensity ultrasound.

The differences between BLM plus 5min US group vs. both sham US and only US is also very significant (p -Value are 0.003, 0.001 respectively) while it shows a very minor significant difference vs. sham MF and only MF groups. It seems that the inhibition effect of pulsed magnetic field on tumor cell growth control is a fact.

According to the results of the only MF group and this fact that magnetic field's doesn't need any interface to enter the body, pulsed magnetic field should be used in every possible center rather than 5 minutes ultrasound to increase the permeability of tumor cells to drug. It is recommended for tumors that are not more than 1cm in depth. In more depth, higher-intensity pulses and re-calculation should be used.

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Authors' Contributions

All authors had equal role in design, work, statistical analysis and manuscript writing.

Conflict of Interest

No conflict.

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