Electro-Molecular Therapy using Adult Mesenchmal Stem Cells

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***Abstract*— Clinically chemo-refractive types of cancers do not respond well to conventional therapies. To treat and enhance the efficacy of drug delivery for these cancers, we have developed an in vitro model of a combination therapy using adult Mesenchymal stem cells. Adult Mesenchymal stem cells have been used for this study primarily because of their ability to home towards tumor cells, making the possibility to practice targeted tumor therapy more realistic. These cells, derived from Human adult bone marrow were subjected to high intensity, short duration (1200V/cm, 100μs), and low intensity, long duration (200V/cm, 40ms and 450V/cm, 25ms) pulses. The effect of these voltages on the viability and proliferation ability of these cells in the presence and absence of Bleomycin (FDA approved chemodrug used for treating various cancers) indicate the possibility of transfer of this technique to clinical practice for effective electro-molecular targeted stem cell therapy. An analysis of the electrical energy applied vs. the viability illustrates a linear relationship. The dose curve exhibits a non-linear relationship. These results indicate that the high efficacy of MSC targeted combination therapy would provide efficient, economical, and enhanced clinical benefit for many types of cancers which need alternate treatments.**

INTRODUCTION

With 556,888 cancer-related deaths in 2006, and approaching 1M now, the necessity for treatment of cancers cannot be overstated. Since cancer is a combination of 100 or more diseases, its treatment also necessitates a combination of therapies as is currently done, with surgery, radiation and chemotherapy. Clinically chemo-refractive cancer patients do not respond well to the current treatment modalities. Hence, the significant need for effective, and economical alternate therapies. Electro-molecular therapy is one such combination therapy where-in high intensity (about 100 to 1000V/cm), short dura-

tion (μs to ms) electrical pulses are applied so as to open-up pores allowing drug/gene/molecular delivery in the otherwise normally impermeable plasma cell membranes. Another attractive attribute of this alternate therapy is its use of very low doses, about $1/10^{th}$ or less of current dosages, hence alleviating the physical pain, side effects as well as economical too [1-4]. With about 50% of all foreclosures and bankruptcies in US due to medical expenses, the economy of cancer treatment is a major issue. Considering that oncology is a very lucrative medical practice, the timeliness and the need for efficient and economical alternate therapy could not be overstated. Towards this end, we have developed an in vitro model of a combination therapy using adult Mesenchymal stem cells. Adult Mesenchymal stem cells possess the ability to home towards tumor cells, making the possibility to practice targeted tumor therapy more realistic [5].

Mesenchymal stem cells are a phenotypically and functionally heterogeneous cell population. In culture, they are defined as plastic-adherent, fibroblast-like cells which are able to self-renew and differentiate into bone, adipose and cartilage tissue. hMSCs were chosen for this study primarily because they share many of the molecular properties of cancer stem cells, and also because they are more abundant and easier to isolate than cancer stem cells [6].

Cancer stem cells (CSCs) are cancer cells (found within tumors or hematological cancers) that possess characteristics associated with normal stem cells, specifically the ability to give rise to all cell types found in a particular cancer sample. CSCs are therefore tumorigenic (tumor-forming), perhaps in contrast to other non-tumorigenic cancer cells (Fig. 1) [7].

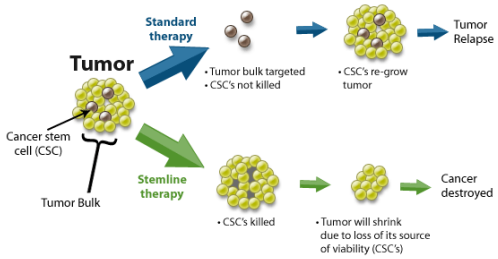


Fig. 1. Importance of Destroying Cancer Stem Cells [7].

CSCs may generate tumors through the stem cell processes of self-renewal and differentiation into multiple cell types. Such cells are proposed to persist in tumors as a distinct population and cause relapse and metastasis by giving rise to new tumors. Therefore, development of specific therapies targeted at CSCs holds hope for improvement of survival and quality of life of cancer patients, especially for sufferers of metastatic disease.

Keeping all this in mind the study concentrates on hMSCs which exhibit characteristics similar to CSCc. To ensure that the medical fraternity readily understands this technqiue, the electroporation parameters are also expressed in terms of energy applied as in a Defibrillator or other medical equipment. In addition, the charge equivalent of the pulse parameters are also analyzed.

Since this method of treatment uses reduced doses of the drug, if offers benefits to the patients in many ways, including physical, economical, and enhanced quality of life.

The main objectives of this study are the optimization of pulse parameters and the selection of optimal dose of bleomycin, the FDA approved chemodrug [8], as the pulse parameters and dosages vary from cell to cell.

MATERIALS & METHODS

A. The Cells - Human Mesenchymal Stem Cells (hMSC)

Human bone marrow aspirates from an adult patient (54 year old male) undergoing cardiac surgery were collected after preinformed consent from the patient and due approval from the Institutional Ethics Committee. They were collected from Sternal bone (Fig. 2) [9] using an aspirating needle. Fig. 3 shows the morphology of an adult hMSCs [10]. The bone marrow sample was carefully overlaid onto the Ficoll Hypaque column (Fig. 4) [10]. The sample was centrifuged at 1800rpm for 20 minutes after a 1:1 dilution with DMEM. The upper layer was aspirated leaving behind the mononuclear cell layer at the interphase. The Buffy coat was transferred to another tube and the volume was made up to 10ml with PBS. The contents were centrifuged at 1800rpm for 10 minutes. After 2-3 washes with PBS, the supernatant was discarded & the pellet was then used for expansion. The cells were seeded at approximately 1 x 10^6cells/mL density in 90 mm cell culture dishes containing low glucose DMEM, 10% FBS, L-Glutamine and antibiotics. The dishes were maintained at 37^{o}C in a humidified atmosphere of 95% air and 5% CO_2. The cells are centrifuged again at 2000rpm for 5 minutes and resuspended to required volume for experimentation.

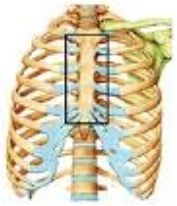


Fig. 2. The sternal bone from which bone marrow is aspirate [9].

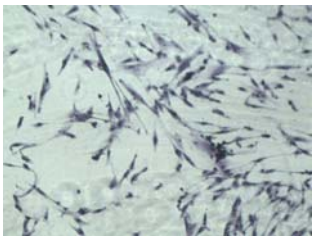


Fig. 3. Adult Mesenchymal Stem cells from a 42 year old male patient [10].

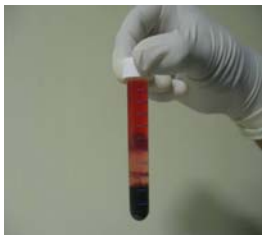


Fig. 4. Ficoll Tube indicating the various layers formation (The top layer is plasma, middle is Buffy coat, and the bottom is red blood cells) [10].

B. The Drug- Bleomycin

Bleomycin, FDA approved in US and by other medical agencies approved and used in the rest of the world ($C_{55}H_{84}N_{17}O_{21}S_3$), is an anti-cancer chemotherapy drug. It is classified as an "antitumor antibiotic". Bleomycin can be used in combination with surgery or radiotherapy, or in the palliative treatment of a number of cancers. It is used in the treatment of squamous cell cancers, melanoma, sarcoma, testicular cancer, Hodgkin's and non-Hodgkin's lymphoma [1-3]. Bleomycin is isolated from culture broth of the fungus Streptomyces verticillus [10]. The Molecular mass of Bleomycin is 1415.55g (The potency of Bleomycin is measured in units of antimicrobial activity. One unit (U) contains 0.56-0.66 mg of Bleomycin and one unit (U) is equivalent to 1000 international units (IU)). Fig. 5 shows the structure of bleomycin.

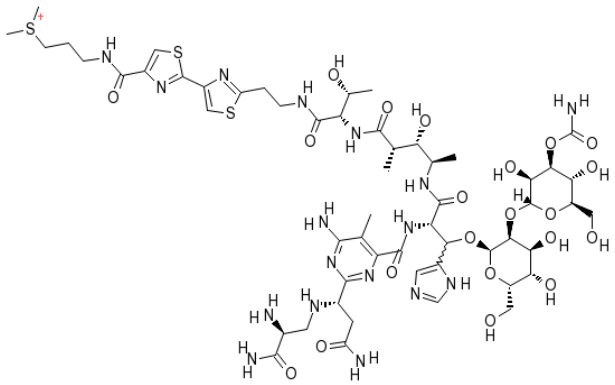


Fig. 5. Structure of Bleomycin.

Bleomycin inflicts direct damage to the DNA. The breaks in the DNA can be seen as chromosomal gaps, deletions and DNA fragmentation. In order to cause DNA damage, bleomycin requires Oxygen and metal to form Fe^{2+}. Bleomycin complex binds to O_2 and then to DNA which results in DNA cleavage. A single molecule of bleomycin can cause around 8-10 DNA breaks which explains the high cytotoxicity of Bleomycin when present inside the cell. Cell death due to bleomycin happens in one of two ways. If only a few thousand bleomycin molecules are present in the cell, the cell is arrested during cell division, becomes enlarged and two or more large nuclei and micronuclei are observed. The cell then dies in a slow process, over one or more days. If instead the cell contains several millions of bleomycin molecules, it is killed within a few minutes. Thus

bleomycin is an extremely toxic agent once inside the cell but this very high intrinsic cytotoxicity is restricted by the inability of bleomycin to freely diffuse through the plasma membrane. It is based on this that the cytotoxicity of bleomycin can be enhanced several times by electroporation [1].

It also causes a number of side effects such as skin rash, hives, changes in skin sensation, hyper-pigmentation (darkening of skin and nails), hair loss, mouth ulcers, hard patches on skin [12] etc. Thus the dose should always be determined based on the patient's response. In our study we aim at studying the effect of reducing the concentration of the drug so as to reduce the side effects and alleviate the pain suffered by cancer victims. The reduction in drug dosage is to be compensated for, by electroporation.

Numerous dose schedules exist for bleomycin and it depends on disease, response and concomitant therapy. For adults the dosage will be weekly or twice weekly and are subjected to 10-20 U/m^2 when the drug is injected intra-venous or intra-muscular or subcutaneous and for intra pleural injection dosage is 60 U/m^2 [13].

C. The Technique - Electroporation

Experiments were carried out with both high Intensity, short duration pulses [1200V/cm, 100µs] and low intensity, long duration pulses (200V/cm, 40ms). In order to study the effect of intermediate values for these parameters, a 450V/cm, 25ms pulse was also used [14]. These pulses were chosen based on previous researchers' and our experiences, as, if the pulse intensity is too high and/or too long, it kills the cells. If it is too low and/or too short, it is insufficient to permeabilize the plasma membrane and does not allow the drug to enter into the cells. Each trial using the above mentioned parameters uses 8 pulses at a frequency of 1Hz.

A BTX ECM 830 (Genetronics, Inc, San Diego, CA), square wave electroporator with 0.4cm (electrode gap) cuvette was used in this study. The electroporation buffer used was DMEM 10% FBS. The sample to be electroporated is taken in the cuvette and is generally fixed at a volume of 200µL. For those experiments requiring electroporation without any drug 100µL of the cell suspension is used along with 100µL of media and electroporated. For electroporation in the presence of the drug 100µL of cell suspension, 50µL of appropriate drug stock and 50µL of media was used. The drug is made up to required concentration by serial dilution technique.

D. The Assay- Cell counting and Viability

After electroporation, cells are counted to determine the viability of cells after each trial. Both live and dead cells are counted. The cells are counted with a dilution factor of 10. 10µL of the solution containing the cells was diluted to 50µL using PBS and mixed homogeneously. 10µL of this is mixed with an equal volume of Trypan blue. 10µL of the thus prepared sample is transferred under the glass cover of a Neubauer hemocytometer (Nexcelom Bioscience LLC. MA) and the cell imaging and counting were done. Trypan blue being an exclusion dye, stains only dead cells blue while the live cells appear as bright spots [15]. The cell viability is estimated for each case from the cell count.

RESULTS & ANALYSES

A. Dose Curve

Most of the chemodrugs have a lifetime quantity that should not be exceeded. For example, not more than 400 units of bleomycin should be adminsitered to a patient in the entire life time. Hence, a dose curve study was performed to identify the efficacy of various doses of the drug and their viabilities for all the three pulse parameters. Fig. 6 the shows dose curve for 200V/cm, 40ms pulse parameters. The viability of cell population decreases with the increase of the dose of the chemodrug. For an order of magnitude change in the dose, the viability varies about 20%, illustrating the non-linear relationship. Fig. 7 shows a comparison of the dose curves of the pulse parameters studied.

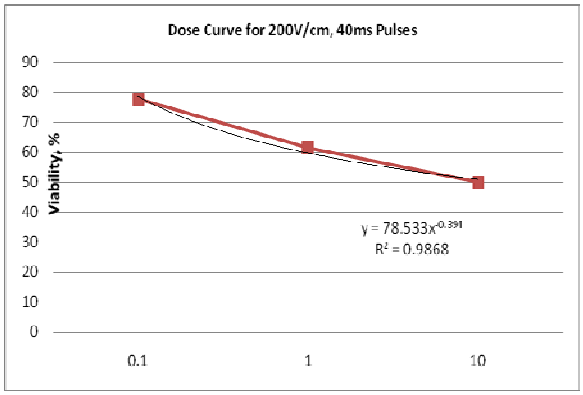


Fig. 6. Dose curve for 200V/cm, 40ms pulse parameters.

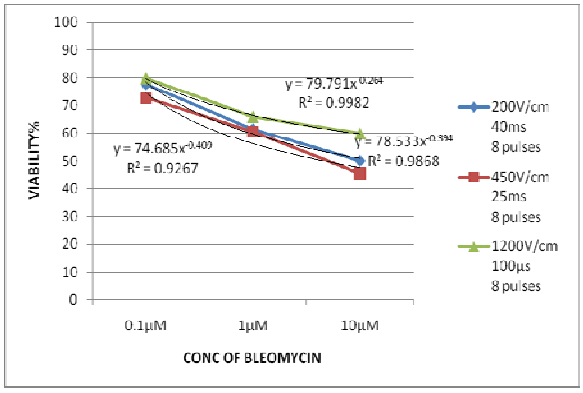
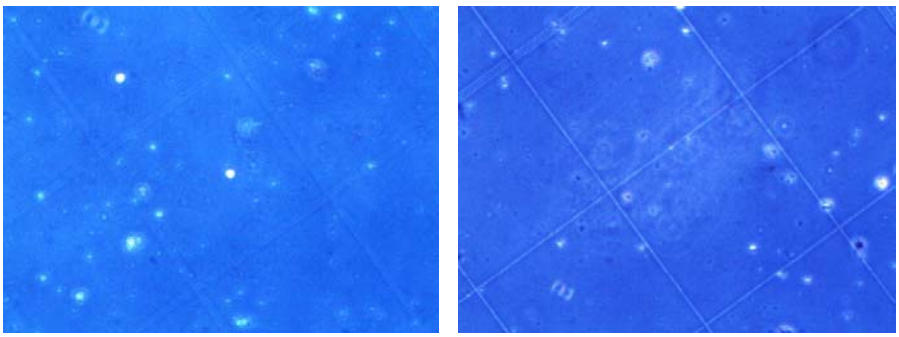


Fig. 7. Dose curve for the three pulse parameters tested.

B. Viability Study

Viability is computed as the ratio of the number of live cells to the total number of cells (live and dead). The percentage viability of the cells under all the experimental conditions are given in Table I. It can be seen that 450V/cm, 25 ms, 8 pulses was the most

intense pulse for all three concentrations of the drug followed by 200V/cm, 40ms, 8 pulses. The 1200V/cm, 100µs, 8 pulses showed the least mortality. This phenomenon can be explained by computing the amount of energy delivered for each pulse set as given below. Fig. 8 shows the live and dead cells using trypan blue exclusion asssy. There are more live cells in the control than in the pulsed cell population at 200V/cm, 40ms, 8 pulses. Similar results were obtained for other pulse parameters (data not shown).



Control 200V/cm, 40ms, 8 pulses

Fig. 8. Trypan blue exclusion assay showing the dead (dark spots) and live (bright spots) cells.

C. Energy Study

Fig. 9 shows the variation of viability with energy applied, V^2TN J/Ω, where V is the applied voltage (E V/cmx0.4cm) in V, T is the pulse width in s and N is the number of pulses. In general, there is decrease in viability with increase in energy applied due to more cell death with more energy applied. Thus a negative linear relationship is obtained for each of the pulse parameter studied. The high value of correlation coefficients indicate good fit. Considering that for a 10fold increase in the BLM dose from 0.1µM to 1µM, the viability only decreased from 77.7% to 61.5%, corresponding to about 20% change, the negative linear relationship for the energy vs viability curve might be due to V squared term in the energy relationship. Thus, energy could be another potential single parameter for electroporation characterization. Similar negative linear relationships were also obtained by other researchers [16, 17].

D. Charge Study

In addition to energy, the viability also depends on the charge, IT = (V/R)T ~ET using the electric field intensity parameter and the time [18, 17]. Here, I is the current in A, T is the pulse width in s, V is the applied voltage and E is the electric field intensity (V/0.4cm). Fig. 10 depicts the results where again a negative linear relationship, similar to that of energy is obtained. The high value of regression coefficient R^2 indicates the goodness of fit and the strong influence of ET on viability. This correlates very well with previous results (19).

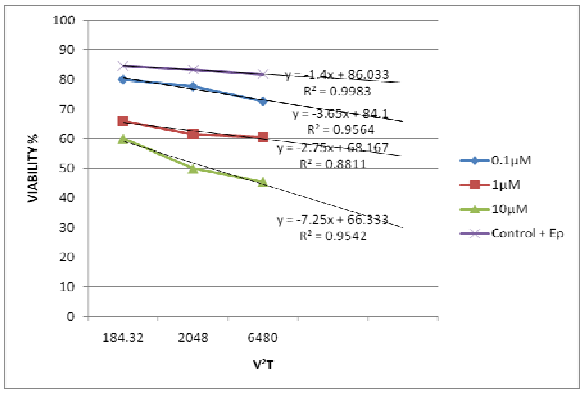


Fig. 9. Energy (J/Ω) vs Viability for the three pulse parameters studied.

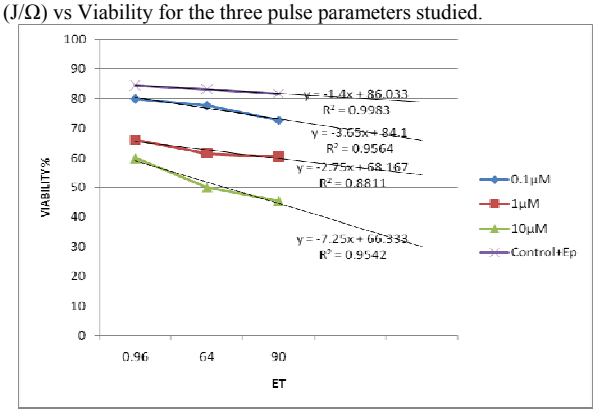


Fig. 10. Charge (C/Ω) vs Viability for the three pulse parameters studied.

TABLE 1: VIABILITY VARIATION FOR VARIOUS STUDY CONDITIONS

Electric field intensity, V/cm	Pulse width, T	Bleomycin Dose	Viability
Control (none)	-	-	93.7%
Bleomycin only	-	1µM	87.0%
EP only 200V/cm	40ms	-	83.3%
EP only 450V/cm	25ms	-	81.8%
EP only 1200V/cm	100µs	-	84.6%
200	40ms	0.1µM	77.7%
450	25ms	1.0µM	72.7%
1200	100µs	10.0µM	80.0%
200	40ms	0.1µM	61.5%
450	25ms	1.0µM	60.5%
1200	100µs	10.0µM	66.0%
200	40ms	0.1µM	50.0%
450	25ms	1.0µM	45.5%
1200	100µs	10.0µM	60.0%

DISCUSSION & SUMMARY

Electrochemotherapy is an extremely effective physical technique that enables cytotoxic drugs to have a direct access to the cytosol [19]. The results of our study indicate that electrical pulses could effectively and economically be used to enhance drug transport across the normally impermeable plasma cell membranes as well as to reduce proliferation in hMSCs. We show here that a single parameter viz. the amount of energy delivered into the cell could be taken into account for designing effective pulse parameters for electro-molecular therapy into stem cells, instead of the three parameters conventionally used, the electric field intensity (or voltage), the pulse width, and the number of pulses. The results reported here correlate well with previous results (Figs. 11a and b [16, 17]) that cell viability decreases in a negative linear fashion with increase in energy delivered. Using energy parameter also has an additional interest since the medical community uses energy values. For example, the energy delivered by a biphasic defibrillator for the resurrection of a patient ranges from 50J to 360J in steps of 50J.

With respect to concentration of the drug it can be clearly seen that as the concentration increases from 0.1μM to 10μM the viability drops drastically in all the cell samples. The variation in the viability is more predominant in higher concentrations than in lower as can be seen from Table I.

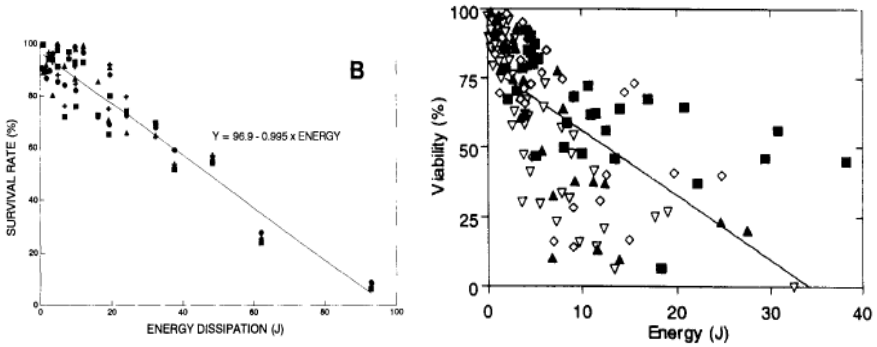


Fig. 11. Negative Linear Relationship between viability vs elctroporation energy applied (a (left), [16], and b (right) [17]).

Considering the trials using 0.1μM concentration, when we move from 1200V/cm to 450V/cm, the viability drops from 80% to 72.7% which is a 9.1% decrease, but the viability for the same parameters at 10μM concentration it drops from 60% to 45.5% which is a 24.1% decrease. By comparing the experiments conducted in the presence and absence of the drug, one can clearly say that the cytotoxicity of the drug is increased greatly in the presence of the pulse than when the drug or electrical pulse alone is administered.

Bleomycin is an antibiotic, used as a cancer chemotherapy agent. It is a cell impermeable drug. Cancer drugs, like bleomycin must enter the cell to be effective, causing single stranded and double stranded breaks in DNA, by production of a free radical complex utilizing O_2 and ferrous iron.

Many potential drugs that have been developed to treat cancer have found limited success due to the lack of efficient and safe delivery systems that allow the molecules to

cross the cell plasma membranes. Electroporation is a viable alternative to upload molecules, including drugs, genes, vaccines, etc.

ACKNOWLEDGMENTS

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