Observing The Bystander Effect Using MCF-7 Cells

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Abstract:

In this study the MCF-7 human breast cancer cell line was used to determine the presence of signals. These signals may be the cause of cell death in untreated cells when both treated and untreated cells are within close proximity to one another. In order to differentiate between the treated and untreated cells, half of the MCF-7s in this experiment were transfected with a Green Fluorescent Protein. The cells that expressed the fluorescent protein were exposed to nanoparticles, followed by an alternating magnetic field. The cells were then co-plated to allow the signals to distribute throughout adjacent cells. The results from this experiment do not corroborate the bystander effect hypothesis with respect to the MCF-7 cell line when used in conjunction with nanoparticle hyperthermia treatment.

Introduction:

The bystander effect refers to the death of cells that have not been treated, but instead are located in close proximity to treated cells, allowing for the formation of gap junction between adjacent cells. The gap junction mediates cell to cell communication in order for the untreated cells to receive signals. These signals are hypothesized to cause apoptosis due to a still largely unknown mechanism.

In the following study half of the population of MCF-7 breast cancer cells will be transfected with a green fluorescent protein. The population that has been transfected will be incubated with 1mg/mL of Fe_3O_4 nanoparticles and placed in an alternating magnetic field. This population will then be co-plated with the naked MCF-7 cell line to determine if the transfected cells have emitted the hypothesized cell death signals.

<u>Background:</u>

Hyperthermia treatment is the use of heat to treat cells, tissues, and/or organisms. The first use of hyperthermia was recorded in the Edwin Smith Surgical Papyrus between 3000 and 2500 B.C. In the past, hyperthermia cancer research was conducted and deemed too invasive for the results produced. However, with advances in nanotechnology, the use of magnetic nanoparticles has allowed for non-invasive treatments via hyperthermia. The nanoparticles used are a form of biocompatible magnetite (Fe_3O_4).

The Iron IV Oxide nanoparticles used in this study were provided by Aduro Biosystems, and manufactured by Micromod through high pressure homogenization according to a core shell method. The particles have an iron oxide core that contains a double coating of dextran with a total hydrodynamic diameter between 80- 130nm. The Fe_3O_4 particles are stored at 4° C at a concentration of 33 mg/mL. When used, they are diluted from a stock solution using sterile phosphate buffered saline. The particles emit heat by hysteresis loss when placed in an alternating magnetic field (AMF).

The equipment that generates different alternating magnetic fields include a generator, coil, cooler, and temperature sensor. The 10kW generator is used at a frequency of 167 kHz to produce an evenly distributed magnetic field at varying Gauss within the coil. The coil is constructed from heated copper tubes molded into a helical structure. The cooler prevents the generator from overheating by maintaining a constant temperature of 30° C. The FISO Command temperature sensor uses a fiber optic probe to record temperatures. When performing a study, the subject in question is place in the coil, within the magnetic field.

These particles are used for both in-vivo and in-vitro studies. In the in-vivo studies tumors are grown in the right flanks of mice to a pre-determined size. The particles are injected either intra-tumoraly or intravenously. Aminated particles are used when injected intravenously. The nanoparticles are also used in conjunction with other cancer treatments such as Chemotherapy, Photo Dynamic Therapy, and Radiation.

Materials:

Implant 200,000 cells into a single well of a six well plate, using 2 ml of transfection media A and incubate for 24 hours at 37° C. Transfection media A contains DMEM media, 10% fetal bovine serum(FBS) and 1% L-Glutamine (percentages are based upon the volume of DMEM used). Since this media lacks Penicillin- Streptomyocin, it is prone to contamination, requiring the entire procedure to be done in a sterile environment.

After the 24 hour incubation, make up transfection media B that contains DMEM media and 1% L-Glutamine. The concentration of GFP cDNA used is 500ng/μL. For this transfection, 1.5 mg of GFP cDNA are required resulting in the use of 3 μL of cDNA solution. Add 100 μL of the transfection media B to the cDNA solution in an eppendorf and mix well using a pipette. In a separate eppendorf add 100 μL of transfection media B to 15 μL of lipofectamine and mix well. Combine both eppendorfs, homogenize, and incubate at room temperature for 30 minutes.

After the incubation, remove the media from the well and replace it with 800 µL of transfection media B. Then add 600 µL of transfection media B to the lipofectamine/cDNA solution. Proceed to add the lipofectamine/cDNA/media solution to the well plate giving rise to a total of 1.4 mL of solution in the well plate. Incubate the well plate at 37° C for 5 hours.

Add 1.4 mL of transfection media B and .28 mL of FBS (20%) to the well. Let the well plate incubate at 37° C for 72 hours. In order to select for GFP expressing cells replace the existing media with MCF-7 selection media. This media includes the same materials as MCF-7 media, but also contains G-418 (geneticin) added at a concentration of 1.5 mg/mL. Use MCF-7 selection media for 4 additional days.

For this experiment, twelve 25 cm^2 cell culture flasks are used in conjunction with one six well plate. The media used is MCF-7 Media; this media contains 500mL of DMEM Medium, 50 mL of Fetal Bovine Serum, 5 mL of L-Glutamine, and 5 mL of Penicillin-Streptomyocin. 0.25% Trypsin is used when disassociating the cells from a culture flask or a well plate. The cells are counted before and after treatment using a Hemocytometer and a Trypan Blue stain that was ordered from Bio Whittake Inc. in Walkersville, MD. The Trypan stain and the sample are added in a 1:1 ratio in a small eppendorf tube. The sample is then loaded into the hemocytometer and counted using a light microscope. A simple calculation is used to determine the number of live cells present in the solution:

Total Cells= (ave. # of cells/sub grid) * (dilution factor of dye) * (total volume in ml the sample was drawn from) * (standard coefficient). The standard coefficient is equal to 1 *10^{4}.

The Cytotox 96® Non-Radioactive Cytotoxicity Assay (LDH Assay) assesses cell death by measuring the presence of lactase dehydrogenase. In this assay 100 µL aliquots of each sample and of sterile media are loaded into a 96 u-bottom well plate in triplicates. A fourth sample is used to determine the total possible cytotoxicity; to this sample 10 µL of lysis solution is added to the well. Once all the samples are loaded place the well plate in a centrifuge for four minutes. Remove 50 µL of each sample's supernatant and place in a flat bottom 96 well plate in the corresponding well. Thaw the assay buffer and add 12 mL of assay buffer to the substrate mix. Once this is mixed, cover the substrate vial with aluminum foil. Add 50 µL aliquots of the substrate mix to each well, cover the plate with aluminum foil, and incubate at 37° C for 30 minutes. Then add 50 µL aliquots of stop solution to each well to stop the reaction. In row H add 150 µL aliquots of sterile phosphate buffered saline to each well. The plate is then read using a standard 96 well plate reader at a wavelength of 490 nm.

Methods:

Using a viable MCF-7 cell line, transfect half of the cell population with the Green Fluorescence Protein (GFP). Once the cells have grown to sufficient quantities, assess the number of both viable naked and GFP expressing cell lines using the Trypan Blue Exclusion Assay. For each cell line label six flasks with the letters A through F and implant each flask with five million cells and five mL of media. Incubate the culture flasks at 37° C for 90 minutes. After incubation, add 1 mg/mL of sterile filtered Fe_3O_4 nanoparticles from the 33 mg/mL stock solution to flasks A, B, C, and F of the GFP culture flasks. Incubate at 37° C for 24 hours.

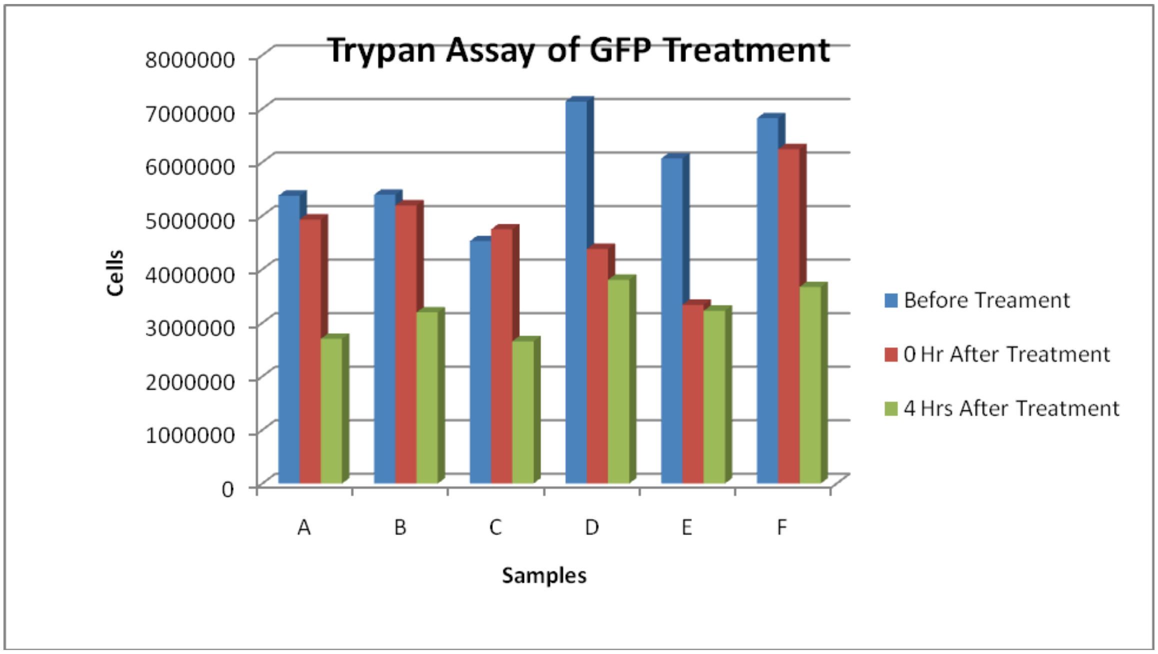
Suction off the solution of media and/or nanoparticles in all the GFP flasks. Wash each GFP culture flask (A-F) with five mL of sterile Phosphate Buffered Saline (PBS). Add one mL of 0.25% Trypsin to each GFP culture flask and wait for ten minutes. Then, add four mL of MCF-7 media to each GFP flask and homogenize the solution; add the five mL solutions of each GFP flask to separate 15 mL conical tubes. Centrifuge each conical tube for ten minutes at 1500 rpm. Suction off the supernatant and break up the pellets. Add 1.5 mL of MCF-7 media to each conical tube and transfer the cell media solutions to 1.5 mL eppendorf tubes labeled A-F respectively. Obtain a 50 µL sample from each eppendorf and use the Trypan Blue Exclusion Assay with a 1:1 ratio to load the sample into a hemocytometer and count the viable cells before treatment. Place the four GFP eppendorf's (A, B, C, & E) into the in-vivo coil at 700 Oe for nine minutes.

Immediately after treatment homogenize each eppendorf and count living cells using the Trypan Blue Exclusion Assay and a light microscope. To evaluate cell death after treatment utilize the Cytotox 96® Non-Radioactive Cytotoxicity Assay (LDH Assay), which is done using a 96-well plate; read the plate using a standard plate reader at 490 nm.

While the assays are running combine the corresponding flasks of naked MCF-7 and GFP cells into a six well plate. Let the well plate incubate for four hours. After the time point assess both cell death and viability using the LDH and Trypan Blue Exclusion Assays.

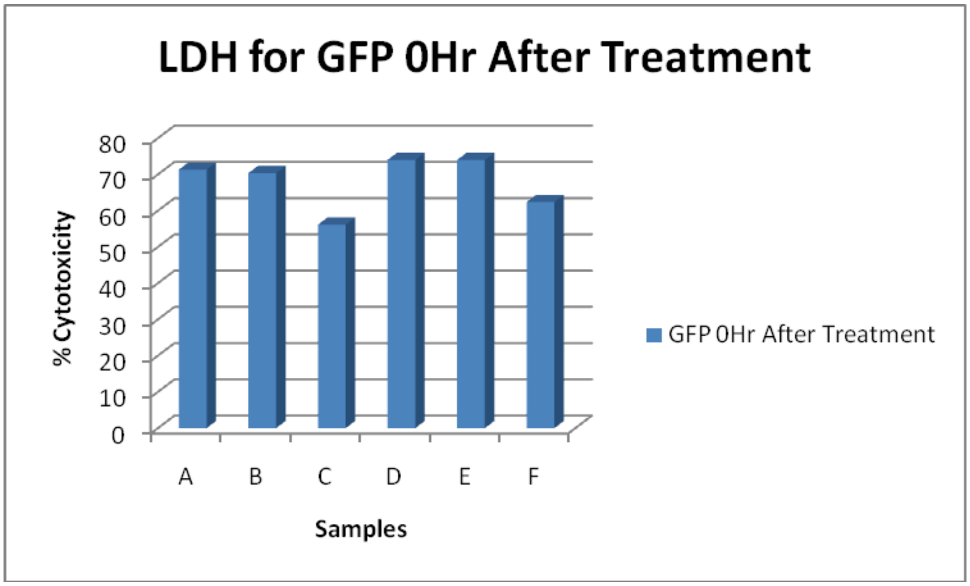
Results:

Figure 1:



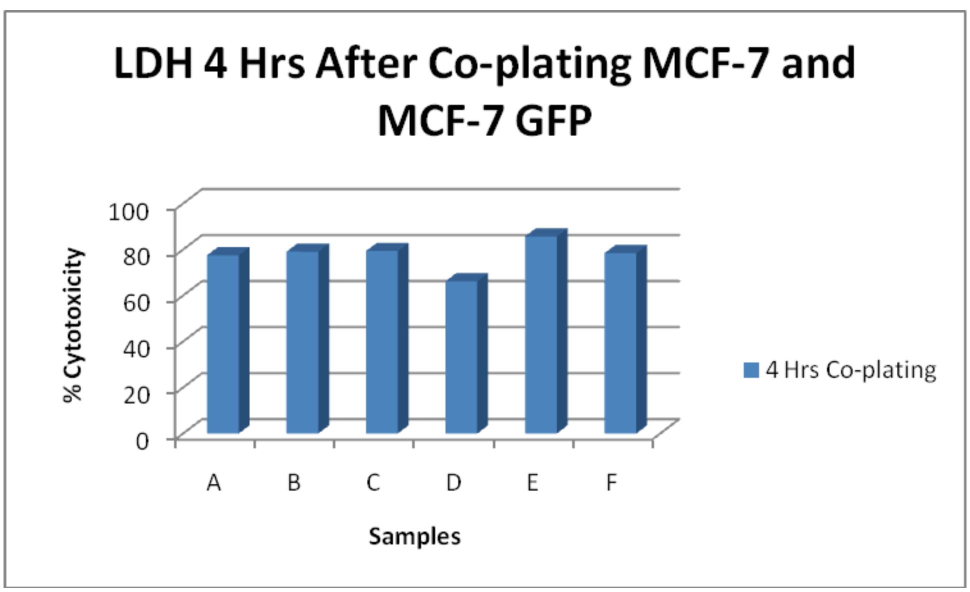
In the above figure, it is observed that the cells that received Fe_3O_4 nanoparticle treatment in conjuction with an AMF had a significant amount of cell death. Samples D and E, the controls, had an inordinate initial cell concentration, and exibited an inexplicable amount of initial cell death after treatment; there seemed to be little cell death after the four hour time point. In samples A, B, C, & F (samples containing nanoparticles), the Trypan assays displayed little immediate cell death, but drastic cell death four hours after treatment.

Figure 2:



The figure above supports Figure 1, displaying highest immediate cell toxicity in samples D and E.

Figure 3:



In this figure, because both naked and GFP MCF-7's were mixed, it is impossible to quantify how many of each cell line died. Figure 3 does correctly show that sample D died the least of all the samples four hours after co-plating.

Discussion:

Reviewing the data gathered, the naked MCF-7 cell line did not exhibit observable signs of cell death, and in fact appeared to replicate at a normal rate. A possible explanation for the lack of cell death could be due to the fact that the treated GFP cells and the naked MCF-7 cells were co-plated 1.5 hours after the prescribed treatment time. The delay in plating was a result of being unable to treat all the samples at once, hindering the simultaneous and immediate mixing of all the MCF-7 and GFP samples. Also, the LDH and Trypan assays had to be conducted from the media of the treated cells before mixing could occur. The 1.5 hour time delay would not have an effect on cell signaling if the cells continuously sent out cytotoxic signals. However, if these signals were sent immediately after death then the set up for this study would be inadequate to assess cell death as a result of short term cell signaling.

Another issue that may have affected the experiment would be the AMF level and treatment time. The magnetic field and time of treatment must be calibrated exactly to induce enough cell death so a large percentage of cells die. A low-powered magnetic field and short treatment time would result in little cell death and not enough cell signaling. On the other hand, too high-powered of a magnetic field and longer treatment time would create excessive cell death before the co-plating could occur, not being the most effective for measuring the transmittance of cell signals.

Conclusion:

The data gathered from this experiment does not directly support the presence of the bystander effect hypothesis in the MCF-7 cell line. The 2 million plated naked MCF-7 cells did not display any signs of cell death; however, the 5 million treated GFP MFC-7 cells displayed a significant amount of cell death after treatment. Future studies may include treating cells at varying: Gauss, treatment times, and incubation times. It would also be advantageous to observe the effects of immediate co-plating over that of delayed co-plating.

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