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In-Vitro Investigations of Nanoparticle Magnetic Thermotherapy: Adjuvant Effects and Comparison to Conventional Heating

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Abstract

Thermotherapy, particularly magnetic nanoparticle hyperthermia, is a promising modality both as a direct cancer cell killing and as a radiosensitization technique for adjuvant therapy. Dextran-coated iron oxide nanoparticles were mixed with multiple tumor cell lines in solution and exposed to varying magnetic field regimes and combined with traditional external radiotherapy. Heating of cell lines by water bath in temperature patterns comparable to those achieved by nanoparticle hyperthermia was conducted to assess the relative value of nano-magnetic thermotherapy compared with conventional bulk heating techniques and data.

Introduction and Background

The use of induced heating to treat pathologies, often referred to as hyperthermia or thermotherapy, is well established in the medical literature. In particular, the use of thermotherapy to treat cancer, either individually or as part of a multi-modality treatment, has gained acceptance in the laboratory and the clinic. Most popularly, thermotherapy is used in conjunction with radiotherapy in order to produce a well-documented radiosensitization effect, increasing the dose effect through multiple possible paths of synergy. Dewhurst et al² and Hildebrandt et al³ provide excellent analyses and summarization of this effect, while Myerson et al's article "Simultaneous superficial hyperthermia and external radiotherapy: report of thermal dosimetry and tolerance to treatment"⁸ exemplifies the relative success and tone of modern clinical investigations.

However, conventional means of heating frequently suffer from the difficulty of limited tissue selectivity, as discussed in depth by Roemer's "Engineering Aspects of Hyperthermia Therapy"⁸ leading to the growing investigations into a new form of heating, nanoparticle magnetic thermotherapy, wherein biocompatible iron oxide nanoparticles are excited with an alternating magnetic field, producing biologically significant levels of heat. Recently, several groups have begun to examine the direct hyperthermic killing and adjuvant effects of nanoparticle thermotherapy *in vitro* and *in vivo* with a variety of biofunctionalization methods, magnetic field characteristics, and combinations with radiological, chemical, and immunological modalities. Akira Ito et al^{5,6} and Masashige Shinkai et al¹⁰ have focused on liposome-encapsulated nanoparticles, particularly their effects on and in conjunction with the immune response, while Ingrid Hilger et al⁴ and separately Andreas Jordan⁷ have thoroughly examined the heating characteristics and cellular uptake rates of dextran-coated and aminosilan-coated nanoparticles. This paper describes several *in vitro* experimental investigations into an under-examined but essential aspect nanoparticle magnetic

thermotherapy – its effectiveness directly compared to conventional heating methodologies as well as the radiosensitization effect of this particular brand of heating.

Materials and Methods

Cell Culture

Two neoplastic cell lines were used in study, one human and one mouse line. MCF-7, a human breast adenocarcinoma was cultivated in 50% Dulcetto's Minimum Essential Media, 50% F-12 Media with Eagle's salts, 10% fetal calf serum, 5% penicillin/streptomycin, and 5% L-glutamine at 37 degrees Celsius. MTG-B, a murine mammary adenocarcinoma, was obtained was likewise cultivated at 37 degrees C in the Alpha modification of Eagle's Minimum Essential Medium with 10% fetal calf serum, 5% penicillin/streptomycin, and 5% L-glutamine. Both cell lines were cultivated in non-pyrogenic cell culture flasks.

Nanoparticles

Two varieties of dextran-coated iron oxide nanoparticles were used in these studies, nominally, 50 nm hydrodynamic radius single-coated iron oxide nanoparticles and 80 nm hydrodynamic radius double-dextran-coated iron oxide nanoparticles, both in aqueous solution and purchased from MicroMod GmbH. The 50 nm particles were obtained at a particle mass concentration of 25 mg/ml, with an iron oxide mass to total particle mass ratio of 0.5, while the 80 nm particles were obtained at a particle mass concentration of 20 mg/ml with an iron oxide mass to total particle mass ratio of 0.44. For the sake of comparability, tests were normalized and described in terms of iron oxide mass concentration.

Magnetic Field Generation

The magnetic fields generated for the study were outputted by a copper solenoid coil conducting a high-frequency alternating electrical current. The electrical current oscillations were generated by a custom capacitor network attached to a power supply. The power supply was a Huttinger TIG 10/300 generator, which enabled real-time adjustment of the system's electrical properties. Magnetic field probe testing confirmed radial homogeneity and vertical dependence of produced fields. Using set physical markers, test samples were consistently located and thus exposed to uniform fields from test to test.

Cell Preparation

Prior to the assorted tests, cells were harvested through the process of trypsinization and counted via methylene blue staining in conjunction with a hemocytometer. Following the removal of excess cultivation flask media, 1 ml Trypsin EDTA was added to each flask, and allowed to react for five minutes whilst the flask was gently rocked. Then, 9 ml of the cell-appropriate media was added to the flask and the mixture was transferred to a centrifuge tube. A 50 microliter sample was withdrawn and mixed with 50 microliter methylene blue this mixture was added to a hemocytometer (Fisher Scientific) and the cell concentration counted under optical microscopy. The primary cell mixture was centrifuged for 10 minutes at 1500 rotations per minute, the supernatant decanted, and the cells physically disrupted into monodispersity. The cells were re-suspended at 1,000,000 cells/milliliter in the appropriate media. Test samples were prepared by transferring 0.2 milliliters of this re-

suspended cell mixture to a 1 ml plastic sterile centrifuge tube, adding and mixing an amount of nanoparticle solution particular to the test, and then adding sufficient media to this to standardize sample volume to 0.3 ml per test tube.

Clonogenic Assay

All test samples were plated for a clonogenic assay after experimentation. According to standard lab practices, each sample underwent serial dilution with the appropriate media and then was plated at two different cell counts, 200 cells/well and 400 cells/well, all in triplicate. The plates were then incubated for 7–10 days at 37 C, decanted of excess media, stained with a methylene blue dye consisting of 1% methylene blue, 10% methanol in a 1x PBS solution for 30 minutes, washed gently with water, and then counted. All counts were normalized to appropriate control branches to account for cell plating efficiency prior to display in the results section.

Isothermal Nano-Magnetic Thermotherapy

Cell samples of MTG-B and MCF-7 were prepared as described above and put on ice to reduce experimental error from nonspecific cytotoxicity at uncontrolled temperatures. The MCF-7 samples were mixed with 50 nm dextran-coated iron oxide nanoparticles at an iron concentration of 3 mg/ml, while the MTG-B samples were mixed with 80 nm double-dextran coated iron oxide nanoparticles at an iron concentration of 2.64 mg/ml. The samples were exposed to a 480 Oe magnetic field oscillating at 146 kHz while thermometry data was recorded by a Luxtron M3000. Once the samples reached a desired temperature, the field strength was manually adjusted between 350 and 450 Oe to maintain the target isotherm within ± 0.3 C for either five or ten minutes. The primary isotherms were 41.5, 43, and 45 Celsius, and all samples reached the desired isotherm in fewer than 10 minutes. Typical heating curves for each of these isotherms are demonstrated in Figure 1. Controls were conducted involving exposing samples of equal volume and cell content with no particles added to the alternating magnetic field as well as control branches wherein cell samples were mixed with particles but exposed to no significant alternating magnetic fields. Following experimentation, all samples were returned to ice and then plated for clonogenic assay.

Isothermal Water Bath Thermotherapy

For the sake of comparison, MCF-7 cell samples with and without 50 nm nanoparticles added at iron concentrations comparable to the above Isothermal Nano-Magnetic Thermotherapy. Said samples were then raised to 30 C in a VWR Scientific Model 1225PC water bath, and then transferred to a Stovall Hybridization Water Bath starting at 30 C and set to attain a desired isotherm of 41.5 or 43 C and a continuous bath mixing. By adjusting the initial water volume of the Hybridization Water Bath and manually adjusting the set goal temperature of the device, this water bath experiment was able to replicate heating curves produced by the nano-magnetic thermotherapy experiments, characterized by a ramp-up of less than ten minutes and then either five or ten minutes at isotherm, ± 0.3 C. Temperature measurements were recorded by a Luxtron M3000 at 15 second intervals. These samples, with the appropriate controls for no treatment, were cooled in ice post-treatment and then plated for clonogenic assay.

Low-Temperature Slow-Pulsing Comparison of AMF and Water Bath Heating

This test, designed to compare the heat damage caused in the region of “ramping up” the temperature of samples from 30 C to 39 C by either nano-magnetic hyperthermia or conventional water bath hyperthermia. MCF-7 samples were prepared with and without 6 mg/ml 50 nm particles, placed in a 30 C VWR Scientific Model 1225PC water bath and then either exposed to a 480 Oe, 146 kHz alternating magnetic field or heated by a Stovall Hybridization Water Bath. Whether by AMF or water bath, samples were increased from 30 to 39 C over a three minute period, and then returned to the 30 C water bath to cool for 15 minutes to 30 C, a cycle referred to as a “slow pulse.” According to experimental branch, samples received between 0 and 10 slow pulses, their temperatures were recorded by a Luxtron M3000, and post-treatment cells were plated for clonogenic assay.

Concentration-Varied Nano-Magnetic Thermoradiotherapy

MTG-B samples were prepared with either a 0, 1, or 5 milligram iron per milliliter solution concentration of 80 nm double-dextran coated iron oxide nanoparticles. The samples were then exposed to either 15 minutes of a 410 Oe, 146 kHz magnetic field, 2 Grey of ionizing radiation, 2 Grey of radiation immediately following 15 minutes of 400 Oe 146 kHz magnetic field, or no treatment. Temperature measurements for the nanomagnetic heating portion of these tests were taken by a Fiso TMI 4, and typical heating over time curves for this test are demonstrated in Figure 2. Samples were kept on ice prior to, in between, and immediately following treatments, and then plated for clonogenic assay.

Results

Isothermal Nano-Magnetic and Water Bath Thermotherapy

As shown in Figure 3, cancerous cells responded significantly to isothermal nanomagnetic and water-bath thermotherapy. Consistently, increasing the temperature or the time of heat exposure increased the cytotoxicity to the cell samples, regardless of treatment modality or cell line. The MTG-B samples displayed higher sensitivity to hyperthermia than the MCF-7 samples at temperatures reaching 45 C, while isotherms of 41.5 and 43 C produced largely similar cytotoxicities in both lines, leaning towards the slightly higher thermal sensitivity of the MTG-B cells at both of these temperatures.

Low-Temperature Slow-Pulsing Comparison of AMF and Water Bath Heating

As shown in Figure 4, water bath and nanoparticle heating by low-temperature slow pulsing produced comparable cytotoxicities at the very lowest and very highest extremes of the number of pulses. For an intermediary number of pulses, however, the nanoparticle heated samples displayed significantly lower survival rates, a gap that reached as much as 22% cell killing for 2 pulses. As the number of pulses increased past this point, the gap narrows and then disappears at the 10 pulse mark, where both modalities produced comparable killing.

Concentration-Varied Nanoparticle Magnetic Thermoradiotherapy

The clearly cumulative and possibly synergistic effects of nanoparticle heating and radiation are visible in Figure 5. The higher, 5 mg/ml concentration of nanoparticles produced

significantly greater cytotoxicity than the 1 mg/ml concentration of nanoparticles when stimulated by an identical alternating magnetic field to produce hyperthermic cell death. The addition of 2 Gy of radiation to the different concentration branches produced a greater amount of cell killing in all three. Furthermore, the relative amount of damage caused by radiation increased as concentration of nanoparticles improved.

Discussion

The above tests reveal a consistent, yet complex picture of the challenges and promise of nanoparticle magnetic thermotherapy. The isothermal data indicated an essentially break-even line where nanoparticle magnetic thermotherapy alone is shown to be at very least comparable to a conventional form of heating in its ability to deliver a steady dose of temperature-correlated killing to cancerous cells. The data also shows the variability between different cell lines in terms of sensitivity to hyperthermic damage — in this case, the MTG-B line was demonstrably, but not radically, more susceptible to the cytotoxic effects of thermotherapy.

The thermoradiotherapy experiment demonstrates nanoparticle magnetic thermotherapy's ability to synergize with an entirely separate modality. While the improved sensitivity of heated cells to radiation and vice versa has been examined and well-proven for other heating methods, this piece of confirmation for nanomagnetic thermotherapy's synergy is important in disproving the possibility of dextran-coated iron oxide nanoparticle composition or binding related somehow interfering with the typical pathways of radiation therapy. Further ahead is also the possibility of investigating the precise characteristics of radiosensitization not only as a function of particle concentration in a sample, but of cocubation and uptake time allowed, different field strengths and frequencies, and activation timing regime.

While the isothermal and thermoradiotherapy data lay the groundwork for the comparability of nanoparticle-mediated magnetic thermotherapy to previous proven methodologies, the slow-pulsing test begins to support the hope that the unique mechanism of this new modality and its as-yet undetermined cytotoxic pathology may be superior to older modalities. In particular, the fact that the nanoparticle-heated particles did more damage to cells during the intermediate number of pulses than the waterbath could, despite the fact that both underwent identical observed temperature patterns, leads us to believe that nanomagnetic thermotherapy may be capable of producing a highly local cytotoxic effect unobservable as a bulk property like a full-sample temperature. It remains to be illuminated whether this sub-global heating damage is characteristic of nanoparticles heating up while merely proximal to cells bound to the surface of cells, or following endocytosis into cells. Should nanoparticle thermotherapy hold promise for producing cell killing at global thermal doses typically considered not significantly cytotoxic, this dramatically increases the potential for using dextran-coated iron oxide nanoparticles in localized low doses such as can be produced by direct intratumoral injection or antibody-targeting to cause tumor-specific damage without bulk heating threatening to damage nearby normal tissues. Alternately, the potential for damage caused by particles not experiencing observable significant heating relative to human body temperatures leads us to underscore the importance of thoroughly

understanding and imaging not only the pathogenesis of nanoparticle magnetic hyperthermia damage, but also the pharmacokinetics and biodistribution patterns of these particles *in vivo*.

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Figure 1.

Sample temperatures under AMF excitation at controlled field strengths to maintain isotherms at 41.5, 43, and 45 C for five minutes.

Figure 2.

Increased temperatures of samples with varying nanoparticle concentration by iron mass during 15 minutes of exposure to a 400 Oe, 146 kHz alternating magnetic field.

Figure 3.

The normalized survival fraction of MTG-B and MCF-7 cells following either 5 or 10 minutes at a listed post-ramp-up isotherm by either nanoparticle magnetic hyperthermia or water bath hyperthermia.

Figure 4.

The normalized survival fraction of MCF-7 cells heated in a series of low temperature pulses with either a water bath or nano-magnetic means.

Figure 5.

The normalized surviving fraction of MTGB cells exposed to 400 Oe for 15 minutes with either 0, 1, or 5 mg/ml iron mass of 80nm dextran-coated iron oxide nanoparticles with or without 2 Gy immediately following heating.