Comparative Efficacy of a Biocompatible Citrate-Functionalized Magnetic Fluid Mediating Radiofrequency Hyperthermia and Magnetohyperthermia to Treat Ectopic Ehrlich-Solid-Tumor-Bearing Elderly Mice

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Abstract

Objective: We aimed to evaluate the efficacy of a biocompatible citrate-functionalized magnetic fluid (NPCit) sample in mediating hyperthermia, using two types of alternated (AC) magnetic field equipment operating in two different ways: a portable apparatus, generating an electromagnetic field in MHz range (radiofrequency range; radiofrequency hyperthermia-RFHT) developed by our research group (CMagMHG), and a commercial one working in kHz range (magnetohyperthermia-MHT) (Magntherm, NanoTherics Ltd, Newcastle, United Kingdom), to treat ectopic Ehrlich-solid-tumor-bearing elderly mice.

Methods: Females intraperitoneally anesthetized with ketamine (80 mg/kg) and xylazine (10 mg/Kg) were subcutaneously inoculated with Ehrlich ascitic tumor cell suspension (1.03 × 10^6 viable cells) in the upper head region for solid form implantation. After 48 hours, mice received one of four treatments: (a) filtered water and no tumor implantation (negative control); (b) tumor inoculation and no treatment (tumor control); (c) intratumoral injection of NPCit (40 µL) containing 18 × 10^13 particles/mL and 13 minutes’ exposure to CMagMHG, operating at 1MHz, 40 Oe field amplitude; (d) intratumoral injection of NPCit and 13 minutes’ exposure to Magnettherm, assembled with 17-turn coil, capacitive box B22, operating under 330 kHz, 4.9 A, 25 V and maximum field strength 170 Oe. Treatments occurred once daily for three consecutive days. Tumor histopathology and semi-quantitative analysis of necrosis area served to assess tumor aggressiveness and regression. Possible acute side effects were assessed by animals’ body and spleen weight and hemogram.

Results: NPCit showed adequate biocompatibility and was effective in mediating RFHT and MHT, which promoted significant increases in necrosis area using both equipment types.

Conclusion: Our findings evidence the potential use of NPCit mediating hyperthermia for future use as an adjuvant in breast cancer therapy.

Keywords: Breast cancer; Citrate-coated maghemite nanoparticles; Magnetohyperthermia; Experimental ectopic breast cancer animal model; Ehrlich tumor; Magnetic fluid

Introduction

Cancer is an important global public health problem [1,2], because the disease is the second leading cause of death in high-income countries (following cardiovascular diseases) and the third leading cause of death in low- and middle-income countries (following cardiovascular diseases and infectious and parasitic diseases) [3]. It accounts for more than six million deaths each year, representing more than 12% of all causes of death worldwide [1,4]; of these, more than 70% of all cancer deaths occur in low and middle-income countries, where the resources available for prevention, diagnosis and treatment are limited or nonexistent [5].

Regarding cancer prevalence, breast, lung, prostate, and colorectal cancers are considered to be the “big four” [6], accounting for about 60% of all cancer cases [7], and even with the discovery of several dozens of novel anticancer drugs (natural and synthetic), the mortality from solid tumor remains constant [8]. This is, in part, because anticancer drugs must gain access to all viable cells within solid tumors in sufficient concentrations to cause lethality [9], if treatment is to be effective. However, solid tumors have a complex microenvironment that includes malignant cells, several types of normal cells and an extracellular matrix [10], besides having a poorly formed blood vascular system with variable rates of blood flow and much larger intercapillary distances than those found in normal tissues [9]. At the same time, most anticancer chemotherapeutic agents act non-specifically, damaging both malignant and normal cells [11,12], which entails acute and chronic adverse effects that can compromise treatment [13]. Furthermore, the use of a single chemotherapeutic antineoplastic drug may show some limitations such as the development of resistance, high toxicity and constraints on clinical procedures [14]; indeed,
conventional antineoplastic chemotherapy has shown low efficiency when the disease is not treated in its early stages [11,12].

In this context, research has endeavored to propose new alternatives and, among them, nanobiotechnology approaches and in particular the use of magnetic nanoparticles (MNPs) have emerged as promising and innovative ways to produce important advances in cancer therapy, such as magnetohyperthermia or magnetic hyperthermia [15]. Biocompatible MNPs coated with biodegradable polymers or molecules may generate heat when submitted to an external alternating current magnetic field, and their use is of interest because they especially target the tumoral tissue, since this is less resistant to sudden increases in temperature than the surrounding normal cells [15-19]. The application of hyperthermia has shown promise as a therapy against cancer [20], and it has been used to enhance the effectiveness of chemotherapy and radiotherapy [21]. This fact could be explained, at least in part, because cells in the S phase of the cell cycle are relatively resistant to radiation, while being sensitive to the increase in temperature. Hyperthermia represents an effective agent for cell destruction, especially for those cells in hypoxia, in need of nutrients and that are in a low pH environment, characteristics commonly present in malignant tumors [22]. Numerous clinical applications with tissue heating have been identified, including reduction in pain or inflammation and preservation of organs [23].

This pilot study aimed to compare the efficacy of a biocompatible citrate-functionalized magnetic fluid (NPCit) sample in mediating hyperthermia using two types of alternated (AC) magnetic field equipment operating in two different ways: a portable apparatus, generating an electromagnetic field in MHz range (radiofrequency range; radiofrequency hyperthermia—RFHT) developed by our research group (CMagMHG), and a commercial one working in kHz range (radiofrequency equipment operating in two different ways: a portable apparatus, generating an electromagnetic field in MHz range (radiofrequency equipment operating in two different ways; a portable apparatus, generating an electromagnetic field in kHz range (radiofrequency equipment operating in two different ways). The first way was by radiofrequency (RF) hyperthermia (RFHT) promoted by a portable apparatus developed by our research group, CMagMHG [27,28], operating at 1MHz with 40 Oe of field amplitude, constituted by one cylindrical metallic rod with 10.7 mm diameter closely wound by a coil of wire concentrating the alternating magnetic flux. The solenoid core is linked to a moveable support to apply the magnetic field in the targeted region (Figures 1A and 1B). The equipment has an oscillating electric circuit with power integrator that generates an alternating electric current at high frequency, and a solenoid at the end of the moveable rod with metal core, which concentrates the electromagnetic field of high frequency generated by the apparatus. At frequencies of the order of MHz, the energy transferred to the nanoparticles promotes heating by Joule effect resulting from eddy currents induced at the interface of the nanoparticles with the liquid medium [29]. The second way to induce hyperthermia was using a commercial piece of equipment, Magnetherm (NanoTherics Ltd, Newcastle, United Kingdom) [30], assembled with 17-turn coil, capacitive box B22, operating under 330 kHz, 4.9 A, 25 V and maximum field strength of 170 Oe. In this device, the animal is introduced inside the coil, keeping it aligned with the coil's main axis (Figure 1C). When subjected to alternating magnetic fields in kHz range, superparamagnetic nanoparticles are heated by the rotation process of the magnetic moment in two conditions, together with the particle in the process, known as Brown rotation, or by the internal rotation independent of the magnetic moment, in what is termed the Neel rotation process. These processes dissipate heat and promote magnetohyperthermia (MHT) [31].

Materials and Methods

Magnetic fluids

To obtain the citrate-functionalized magnetic fluid (NPCit), nanoparticles of magnetite were synthesized by co-precipitation of Fe (II) and Fe (III) in alkaline medium, oxidized to maghemite ($\gamma$-Fe$_2$O$_3$) [24], and then stabilized by citrate. The pH was adjusted to 7.0 and the salinity to 0.9% to promote biocompatibility [25,26].

With the aim of determining structure and nanoparticle size, we performed X-ray power diffraction (XRD) using a Shimadzu diffractometer model DOX-6000 with copper CuKα radiation, $\lambda=1.54$ Ångstrom at 40 kV and 20 mA. For this, the sample was dehydrated in a low vacuum environment and stored as a powder without atmospheric contact; using a Zetasizer (Zetasizer Nano-ZS90 Malvern Instruments Limited, Malvern, UK) we determined the hydrodynamic radius, the polydispersion index and its surface charge by dynamic light scattering (DLS) with the sample diluted in ultra-pure deionized water (1:500), and the measurements were performed in triplicate at 25°C, with a fixed detection angle of 173°C; the physical mean diameter and its dispersion was determined by transmission electron microscopy (TEM) performed on a JEOL 1011 (JEOL USA, Inc.) with sample diluted in ultra-pure deionized water (1:500), deposited in a 300 mesh copper TEM grid supported by films of Formvar, dried at room temperature for 24 hours and then counterstained for 45 minutes with 1% Osmium (OsO$_4$) vapor. The average diameter of the sample evaluated by MET was obtained by computer analysis, using Image-ProPLus software version 5.1.

Hyperthermia equipment

To induce hyperthermia, two types of equipment generating an AC magnetic field were used, operating in two different ways. The first way was by radiofrequency (RF) hyperthermia (RFHT) promoted by a portable apparatus developed by our research group, CMagMHG [27,28], operating at 1MHz with 40 Oe of field amplitude, constituted by one cylindrical metallic rod with 10.7 mm diameter closely wound by a coil of wire concentrating the alternating magnetic flux. The solenoid core is linked to a moveable support to apply the magnetic field in the targeted region (Figures 1A and 1B). The equipment has an oscillating electric circuit with power integrator that generates an alternating electric current at high frequency, and a solenoid at the end of the moveable rod with metal core, which concentrates the electromagnetic field of high frequency generated by the apparatus. At frequencies of the order of MHz, the energy transferred to the nanoparticles promotes heating by Joule effect resulting from eddy currents induced at the interface of the nanoparticles with the liquid medium [29]. The second way to induce hyperthermia was using a commercial piece of equipment, Magnetherm (NanoTherics Ltd, Newcastle, United Kingdom) [30], assembled with 17-turn coil, capacitive box B22, operating under 330 kHz, 4.9 A, 25 V and maximum field strength of 170 Oe. In this device, the animal is introduced inside the coil, keeping it aligned with the coil's main axis (Figure 1C). When subjected to alternating magnetic fields in kHz range, superparamagnetic nanoparticles are heated by the rotation process of the magnetic moment in two conditions, together with the particle in the process, known as Brown rotation, or by the internal rotation independent of the magnetic moment, in what is termed the Neel rotation process. These processes dissipate heat and promote magnetohyperthermia (MHT) [31].

In vivo tests

Ehrlich tumor: The Ehrlich ascitic tumor was maintained in ascitic form by passages in Swiss mice, by weekly intraperitoneal transplantation of 10$^6$ tumor cells [32]. The ascitic fluid, collected by intraperitoneal puncture, was counted in a Neubauer hemocytometer, and the Trypan blue dye exclusion method was used to evaluate the viable cells [33].

Animals and experimental design

Elderly female Swiss albino mice, 10-11 months, obtained from the Multidisciplinary Center for Biological Investigation in Laboratory Animal Science (CEMIB) of the State University of Campinas (Unicamp, SP/Brazil), were initially housed in plastic cages (6 animals/cage) at room temperature (22°C ± 2°C) in a 12 h light/dark cycle with lights on at 6 a.m. and free access to food and water.

After being acclimatized to laboratory conditions for two weeks, animals, weighing 45.6 g ± 5.47 g at the beginning of the experiment, were randomly distributed in the treatment and control groups (N=3/group), following the guidelines for the design and statistical analysis of experiments using laboratory animals for a pilot study [34], according to the recommendations of the international practices for animal use and care, and the Animal Ethics Committee of the Institute of Biological Sciences of the University of Brasilia/Brazil. For the xenograft, animals were intraperitoneally anesthetized with ketamine (80 mg/kg) and xylazine (10 mg/kg) and subcutaneously inoculated with 40 μL of previously frozen Ehrlich ascitic tumor cell suspension containing 1.03 x 10$^6$ viable cells, in the upper region of the head for solid form.
implantation [15,32,35,36]. Following 48 hours after xenograft, all mice had clinical tumor and received the following treatments: (a) filtered water and non-tumor-bearing mice (negative control); (b) xenograft and no treatment (tumor control); (c) intratumoral injection of NPCit (40 µL) containing $18 \times 10^{18}$ particles/mL and exposure for 13 minutes to CMagMHG; (d) intratumoral injection of NPCit and exposure for 13 minutes to Magnetherm. Treatments were performed once a day for three consecutive days [15,19,35]. Body weights were measured at the beginning and end of the treatment, and the animals were monitored for signs of toxicity such as weight loss, diarrhea, skin ulcers and deaths.

Twenty-four hours after the last treatment, mice were anesthetized with ketamine and xylazine according to the method described above. Blood samples (400 µL) collected by cardiac puncture were used to carry out hemogram in a multiple automated hematology analyzer for veterinary use, Sysmex pcoH-1000 Diff (Curitiba/Paraná, Brazil) calibrated for mice in microtubes containing EDTA as anticoagulant. Euthanasia of the animals was carried out by cervical dislocation according to the Guidelines on Euthanasia of the federal council of veterinary medicine (CFMV) [37]. Tumors and spleens were surgically removed and weighed, the tumors’ width, length and thickness were measured using a digital pachymeter, and their volume was calculated according to Yanase et al. [38]. Thereafter, they were fixed with 10% formalin for 24 h, transferred to 70% ethanol, embedded in paraffin using an automatic tissue processor (OMA® DM-40, São Paulo, Brazil), cut to 5 µm of thickness in a Leica RM2235 manual microtome (Leica Microsystems, Nussloch, Germany) and stained with Hematoxilin-Eosin (H&E) according to standard procedures, for histological analyses (light microscopy). For iron detection, Prussian Blue staining was performed by the Perl's method, followed by counterstaining with Nuclear Fast Red [39]. All histological sections were photographed with an MC 80 DX camera coupled to a Zeiss Axiophot light microscope. Tumor necrosis was evaluated using a semi-quantitative method. A semi-quantitative score (0-4: 0 for none, 4 for 100% of necrosis) was used to determine the extension of necrosis area. At least five images of each microscopy field (microscope 20x magnification) were analyzed in 5 different histological slides, with a total of 25 histological tumor fields analyzed for each experimental individual.
Tumor volume, histopathology and semi-quantitative analysis of necrosis area were used to assess its aggressiveness and regression. Possible acute side effects were assessed by animals’ body and spleen weight and hemogram.

All animal handling and procedures were carried out according to the international practices for animal use and care, and approved by the Animal Ethics Committee of the Institute of Biological Sciences of the University of Brasilia/Brazil, reference number 44783/2013.

Statistical Analysis

Statistical analysis was carried out using SPSS 17.0 or GraphPad Prism 5.0, and values of p<0.05 were considered significant. The continuous variables were tested for normal distribution with Shapiro-Wilk. Possible differences among the groups were investigated through ANOVA or the Kruskal-Wallis test (data normally distributed), followed respectively by Tukey’s or Dunn’s Multiple Comparison Tests.

Results

Physicochemical characterization of the citrate-functionalized magnetic fluid (NPCit)

NPCit showed brown color, with iron concentration of 16.8 mg/mL and estimated maghemite nanoparticle concentration of 18 × 10^18 particles/mL. Magnetic nanoparticles (MNPs) diameters measured by the three different techniques showed average size values of 8 nm (diameter of the crystalline structure) by X-ray diffraction (XDR); 14.28 (modal average diameter) by transmission electronic microscopy (TEM); and 66.34 nm (hydrodynamic average diameter) by dynamic light scattering (DLS). The average polydispersion index (PDI) and surface charge measured by the zeta potential were 0.24 and -42.1 mV, respectively (Figure 2).

In vivo tests

Tumor histopathology and measurements: Areas of viable tumor cells surrounding central areas of necrosis were seen in all experimental groups (Figures 3A and 3B). In the treatments with hyperthermia, the presence of MNPs was observed in the tumor area (Figure 3C), as was phagocytosis of them (Figure 3D). NPCit showed adequate biocompatibility and was effective in mediating hyperthermia (RFHT and MHT), which promoted non-significant reductions in tumor volume but significant increases in necrosis area, using both types of equipment (Figures 3E and 3F).

Evaluation of the acute side effects: No significant differences were found in the animals’ body and spleen weight between the groups, but there was a tendency to greater spleen weight with treatments with hyperthermia, mainly using MHT (Magnetherm equipment). Also, no skin ulceration, weight loss or evident alterations were found, indicating the good tolerance of mice to the treatments.

Regarding results of the hemogram, the only statistically significant difference observed was an increase of neutrophils+monocytes (%) in both treatment groups, with RFHT (CMagMHG) and MHT (Magnetherm), compared to negative control (Table 1).
Discussion

Nanometer-sized particles have attracted much attention, not only because of their unusual properties, but also due to their potential variety of applications in industry and in biomedical applications such as controlled drug delivery, nuclear magnetic resonance imaging agents, magnetic field guided drug transporters, tumor treatment via hyperthermia, magnetic biomolecular separation and diagnosis [40-43]. Surface-functionalized superparamagnetic iron oxide nanoparticles (SPIONs) are usually based on magnetite (Fe₃O₄) or maghemite (γ-Fe₂O₃) nanoparticles [15,17,44,45], and represent a promising platform to build complex magnetic systems used for in vivo applications [35,46]. Since particle size and surface charge are important factors that determine their pharmacokinetics, toxicity and biodistribution [35,47], they must have adequate magnetic characteristics besides control in size and surface composition [43], because their physical characteristics can affect their in vivo performance [35,47].

Nanoparticles (NPs) composed of ferromagnetic materials and with
Even though clinical tumor volume was non-

\[18,19\]. In this context, NPcit showed adequate biocompatibility and are therefore capable of producing localized heating in a desired area. The cytotoxicity of NPs depends, among other things, on surface charge; NPs with positive charges are more toxic than negatively charged particles \([35,50]\). NPs with zeta potentials greater than +30 mV are considered approximately neutral. As most cell membranes are negatively charged, zeta potential may affect the tendency of a NP to permeate membranes, with cationic particles generally exhibiting a more toxic association with cell wall rupture \([31]\). Based on the zeta potential result, we can suppose that the NPCit will maintain a negative surface charge in the physiological pH, corroborating a previous study carried out with healthy animals \([28]\), and the installation of the Ehrlich solid tumor by itself has been demonstrated by our group as non-significantly decreasing lymphocytes and significantly increasing neutrophils+monocytes observed for both treatments, particularly with CMagMHG, corroborate this suggestion.

CMagMHG was developed in order to perform hyperthermia tests, when biological tests were conducted after exposing the peritoneal region of mice to the AC field. This work showed that the damage induced by the AC field to normal cells is related to the exposure time, and (2) the equipment developed is appropriate to perform hyperthermia experiments. Significant alterations of increased lymphocytes were observed, simultaneously with decreased monocyte frequency, when the peritoneum was exposed for 10 minutes, not observing alterations in the neutrophil frequency at any time after AC field exposure, an important signature of inflammatory reaction \([28]\). Although these results appear contradictory in respect to ours, this study was carried out with healthy animals \([28]\), and the installation of the Ehrlich solid tumor by itself has been demonstrated by our group as non-significantly decreasing lymphocytes and significantly increasing neutrophils+monocytes \([32]\), corroborating our results. Even if the 10 minutes of magnetic field exposure and treatments used were previously tested \([15,19,28,35]\) and have been chosen to be compared to normal cells is related to the exposure

| Erythrogram | Negative control | Tumor control | CMagMHG | Magnetherm | p-value |
|-------------|-----------------|--------------|---------|------------|---------|
| RBC (x 10^12/L) | 7.02 ± 2.48 | 9.12 ± 0.35 | 7.39 ± 1.43 | 8.71 ± 0.32 | 0.434 |
| HGB (g/dL) | 9.98 ± 3.55 | 13.60 ± 0.00 | 10.77 ± 1.79 | 12.43 ± 0.95 | 0.344 |
| HCT (%) | 24.93 ± 8.96 | 33.80 ± 1.70 | 26.70 ± 4.78 | 33.57 ± 3.90 | 0.266 |
| MCV (fL) | 35.50 ± 0.74 | 36.55 ± 0.35 | 36.20 ± 0.62 | 38.50 ± 3.99 | 0.535 |
| MCH (pg) | 14.25 ± 0.58 | 14.70 ± 0.85 | 14.63 ± 0.51 | 14.30 ± 0.61 | 0.757 |
| MCHC (g/dL) | 40.18 ± 0.90 | 40.30 ± 1.98 | 40.40 ± 0.95 | 37.24 ± 4.29 | 0.360 |
| RDW (%) | 14.93 ± 0.59 | 14.15 ± 0.21 | 14.90 ± 0.36 | 14.70 ± 0.26 | 0.231 |

| Leukogram | Negative control | Tumor control | CMagMHG | Magnetherm | p-value |
|-----------|-----------------|--------------|---------|------------|---------|
| WBC (x 10^12/L) | 2.23 ± 0.67 | 2.25 ± 1.20 | 3.17 ± 0.67 | 2.53 ± 0.90 | 0.542 |
| Lymphocytes (%) | 52.97 ± 12.65 | 51.80 ± 2.83 | 34.37 ± 7.31 | 45.20 ± 7.28 | 0.129 |
| Neutrophils+Monocytes (%) | 35.73 ± 6.04 | 45.30 ± 0.42 | 62.97 ± 7.31** | 52.97 ± 6.23** | 0.005 |
| Eosinophils (%) | 3.40 ± 3.11 | 2.90 ± 2.40 | 2.67 ± 1.04 | 2.75 ± 1.06 | 0.977 |

| Plateletgram | Negative control | Tumor control | CMagMHG | Magnetherm | p-value |
|--------------|-----------------|--------------|---------|------------|---------|
| PLT (x10^12/L) | 1115.00 ± 492.40 | 1036.00 ± 179.61 | 969.67 ± 240.56 | 1250.67 ± 164.93 | 0.735 |

Data correspond to mean ± standard deviation (SD). RBC=Red Blood Cells; HGB=Hemoglobin; HCT=Hematocrit; MCV=Mean Corpuscular Volume; MCH=Mean Corpuscular hemoglobin; MCHC=Mean corpuscular hemoglobin concentration; RDW=Red cell distribution width (represents an indication of the amount of variation-anisocytosis-in cell size); WBC=Total White Blood Cells; PLT=platelet count; MPV=mean platelet volume; g/dL=grams per deciliter; fl=fentoliters; pg= picograms. P-values are generated by ANOVA. The superscript letters indicate significant differences detected by the Tukey’s post hoc test, with *a=significant compared to negative control. Asterisks indicate significant differences at *p<0.05 and **p<0.01.
was already expected, since the animals are exposed as a whole to the magnetic field in Magnetotherm, while in CMAcMHHG, the magnetic field was applied to the targeted region (tumor) (Supplementary Figure).

Conclusion

NPcit showed adequate biocompatibility and was effective in mediating RFHT and MHT, which promoted non-significant reductions in tumor volume but significant increases in necrosis area in mediating RFHT and MHT, which promoted non-significant

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