Characterization of intratumor magnetic nanoparticle distribution and heating in a rat model of metastatic spine disease

Laboratory investigation

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Object. The goal of this study was to optimize local delivery of magnetic nanoparticles in a rat model of metastatic breast cancer in the spine for tumor hyperthermia while minimizing systemic exposure.

Methods. A syngeneic mammary adenocarcinoma was implanted into the L-6 vertebral body of 69 female Fischer rats. Suspensions of 100-nm starch-coated iron oxide magnetic nanoparticles (micromod Partikletechnologie GmbH) were injected into tumors 9 or 13 days after implantation. For nanoparticle distribution studies, tissues were harvested from a cohort of 36 rats, and inductively coupled plasma mass spectrometry and histopathological studies with Prussian blue staining were used to analyze the samples. Intratumor heating was tested in 4 anesthetized animals with a 20-minute exposure to an alternating magnetic field (AMF) at a frequency of 150 kHz and an amplitude of 48 kA/m or 63.3 kA/m. Intratumor and rectal temperatures were measured, and functional assessments of AMF-exposed animals and histopathological studies of heated tumor samples were examined. Rectal temperatures alone were tested in a cohort of 29 rats during AMF exposure with or without nanoparticle administration. Animal studies were completed in accordance with the protocols of the University Animal Care and Use Committee.

Results. Nanoparticles remained within the tumor mass within 3 hours of injection and migrated into the bone at 6, 12, and 24 hours. Subarachnoid accumulation of nanoparticles was noted at 48 hours. No evidence of lymphoreticular nanoparticle exposure was found on histological investigation or via inductively coupled plasma mass spectrometry. The mean intratumor temperatures were 43.2°C and 40.6°C on exposure to 63.3 kA/m and 48 kA/m, respectively, with histological evidence of necrosis. All animals were ambulatory at 24 hours after treatment with no evidence of neurological dysfunction.

Conclusions. Locally delivered magnetic nanoparticles activated by an AMF can generate hyperthermia in spinal tumors without accumulating in the lymphoreticular system and without damaging the spinal cord, thereby limiting neurological dysfunction and minimizing systemic exposure. Magnetic nanoparticle hyperthermia may be a viable option for palliative therapy of spinal tumors.

(key Words • magnetic nanoparticles • iron oxide nanoparticles • spine • tumor • rat • hyperthermia • oncology)

It is estimated that in the year 2020, 15 million people worldwide will receive a diagnosis of cancer. 10 Breast, lung, and prostate neoplasms are the most commonly diagnosed cancers, and these are the most likely to metastasize to the osseous spine. 22,27 Metastatic spine lesions occur in 30%–90% of these patients, 35,36 compromising their quality of life with debilitating symptoms that include intractable pain and deficits in sensory, motor, or autonomic neurological function. 1,8,17,22,41,42 Improved treatment of advanced-stage cancer has led to more patients who are living with systemic disease, thus increasing the number of people living with lesions of the spine. Patients who suffer from metastatic spine disease can initially present with a high systemic tumor burden that...
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...may contraindicate traditional therapies. Depending on the underlying tumor pathology, current treatment options include a combination of resection, focused radiation therapy, or systemic chemotherapy. Although resection can be effective for local tumor control, patients with metastatic disease are often contraindicated for aggressive resection. Conventional chemotherapy is systemically administered and can have significant side effects such as bone marrow suppression as well as hepatic and renal dysfunction due to damage of normal tissue. Targeted local therapy is emerging as a method to destroy tumor tissue selectively while minimizing systemic effects for a select patient population.

Heat is a potent therapeutic agent that effectively sensitizes cancer to the effects of ionizing radiation. It can also provide palliation for many tumor types because its broad mechanisms of action are unlike the targeted or DNA-damaging effects of chemotherapies and radiation. Its tumoricidal effects therefore are less dependent on specific tumor types and are less susceptible to acquired resistance. Because heat is mechanical incoherent energy that does not discriminate by tissue type, technological barriers have thwarted development of an effective delivery method for cancer-specific heat therapy, or hyperthermia, precluding widespread clinical application. Considerable research has been devoted to developing sources of electromagnetic, laser, and ultrasound energy for cancer-specific hyperthermia. Although modest success can be claimed, widespread clinical adoption of thermal technologies for cancer therapy is not a reality, despite the significant biological and physiological advantages its use offers.

A renewed interest in hyperthermia has been spurred by recent progress in nanotechnology to produce nanometer-scale energy-susceptive materials; that is, materials that efficiently couple with an energy source to deposit heat locally. Among the materials and technologies that have been proposed, ferromagnetic nanoparticles coupled with alternating magnetic fields (AMFs) have emerged as particularly encouraging. Magnetic nanoparticles can be suspended in biocompatible carrier media and anatomically targeted to tumors by direct delivery via intratumor injection. Magnetic materials dissipate heat from relaxation losses when they are exposed to an AMF. The extent of power loss or heating depends on the structural and magnetic properties of the magnetic material, and on the frequency and amplitude of the AMF. Magnetic nanoparticle heat therapy thus offers potential for both precise delivery of heating because their small size enables access and penetration throughout tumor tissues, and controlled dose deposition through control of the AMF power settings. Furthermore, direct and intratumor delivery of particles minimizes systemic exposure of the patient to the magnetic material, thereby enhancing safety because activation with AMF is a noninvasive procedure. For palliation of spinal metastases, this precision and noninvasive activation and control is critical to avoid damaging adjacent heat-sensitive neural tissue while still ensuring a minimum effective dose throughout the tumor.

To our knowledge, magnetic nanoparticle hyperthermia for palliation of spinal metastases has not been explored. Using an established rat spine tumor model, we describe methods to deliver a biocompatible suspension of magnetic iron oxide nanoparticles that achieve widespread intratumoral particle distribution. Total nanoparticle concentration in tissues was characterized by quantifying iron concentration using inductively coupled plasma mass spectrometry (ICP-MS), and intratissue distribution of particles was assessed by histopathological investigation following staining with Prussian blue (PB) and H&E. Cohorts of animals were exposed to AMF for 20-minute periods, followed by histopathological investigation to assess the extent of intratumor and systemic heating and local thermal damage to tissues. The presence of nanoparticles in tumors that were exposed to AMF correlated with significantly elevated tumor temperatures, whereas rectal temperatures remained tolerable. Necrosis could be seen in nanoparticle- and AMF-treated animals. Conversely, a significant increase of iron from nanoparticles was not observed in CSF or lung, liver, and spleen tissues. These results demonstrate the feasibility of AMF-mediated selective tumor heating by injecting magnetic nanoparticles of iron oxide into spinal tumors. Additional research and development is warranted.

Methods

Nanoparticle Suspension

Suspensions of starch-coated magnetite (Fe$_3$O$_4$) core-shell particles (Bionized Nanoferrite [BNF], catalog no. 10–00–102) were obtained from micromod Partikeltechnologie GmbH. Synthesis, structure, and magnetic properties have been previously described, and a summary is provided. These nanoparticles were produced by precipitating ferric and ferrous sulfate salts from solution with high pH in a high-pressure-homogenization reaction vessel. The iron content and mean hydrodynamic radius, measured by photon correlation spectroscopy, were provided by the manufacturer. The iron content was reported as > 70% w/w, with a total iron concentration of approximately 30 mg Fe/ml (42 mg particles/ml). The particles were suspended in sterile water to provide a stable biocompatible suspension having a mean hydrodynamic diameter of approximately 100 nm.

Specific Loss Power Characterization

The amplitude-dependent heating rate, or specific loss power (SLP), for particles was estimated from measured time-dependent heating in the AMF device (described below) at several applied amplitude (voltage) values. Sample temperatures were measured with fiber-optic probes (FISO Technologies) at 1-second intervals. The SLP was estimated from the slope ($\Delta T/\Delta t$) of the time-temperature curve by using methods previously described.

Briefly, a 1-ml volume of nanoparticle suspension was placed in a standard 12-mm polystyrene test tube and inserted into the insulating sample holder. Equilibrium between the probe, sample, and the calorimeter was confirmed and the AMF power was applied. At each power...
setting a sample of distilled water was measured to correct for calorimeter heat capacity.

The temperature at the time interval \(T_0\) was subtracted from the initial temperature \(T_1\) to yield the net temperature change: \(\Delta T = T_1 - T_0\). This process was repeated for water blank samples. The resulting net water blank temperature change was subtracted from that of the sample at each point to yield the corrected temperature change for each sample. The SLP was estimated from the initial and steepest part of the slope \(\Delta T/\Delta t\) of the time-temperature curve, by fitting a linear weighted least-squares function (Origin) to the data. The appropriate interval for calculating the slope was determined by analyzing a plot of the incremental temperature change, analogous to the first derivative of the heating rate (for a complete description of the procedure, please see Bordeleon et al.\(^6\)).

**The AMF System**

The AMF system has been previously described,\(^6,7\) and it comprises 4 main components: a power supply, an external impedance matching network, a modified solenoid coil as the inductor, and a closed-circuiting water cooling system. The power supply is an 80-kW induction heating system manufactured by PPECO that provides an alternating current to a resonant circuit with variable frequency between 135 kHz and 440 kHz. The external impedance match network (AMF Life Systems, Inc.) was adjusted for stable oscillation at 150 ± 1 kHz.

Detailed descriptions of the design, build, and characterization of the modified solenoid coil have been published previously.\(^6,7,33\) Briefly, when combined the system is capable of producing a homogeneous flux density AC magnetic field (at 150 kHz) in a cylindrical volume having a 5-cm diameter with a > 6-cm length, for peak-to-peak amplitudes between 4 kA/m and 95 kA/m. The field amplitude can be dynamically controlled by adjusting the power supply output voltage. For rectal thermometry experiments, the AMF amplitude was fixed at 48 kA/m, and animals were exposed for a 20-minute duration. For intratumoral thermometry at 48 kA/m and 63.3 kA/m, the average time of exposure was 26 and 27.5 minutes, respectively. The magnitude of the magnetic field was measured prior to animal exposure at the center of the solenoid by using a magnetic field probe.\(^7\)

**Cell Line and Culture**

The rat mammary adenocarcinoma CRL-1666 (13762 MAT B III) cell line used in this study was purchased from the American Type Culture Collection, and cells were maintained in both tissue culture and as solid tumor. In culture, cells were loosely adherent and were maintained in McCoy 5A medium, modified with 10% fetal bovine serum, streptomycin (80.5 pg/ml), penicillin (base, 80.5 U/ml), and 1% L-glutamine (all products from Gibco Laboratories) in a humidified atmosphere containing 5% CO\(_2\) and maintained at 37°C. Cells were grown to a concentration of 1 million cells/ml and then resuspended in medium. Resuspension was repeated approximately every 4 days.

**Animal Studies**

Sixty-nine female Fischer rats (National Cancer Institute, Frederick National Laboratory) were used in this study. All were 6–8 weeks old and weighed 150–200 g prior to treatment. Rats were housed in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care in compliance with the Guide for the Care and Use of Laboratory Animals, and all procedures were approved by the Institutional Animal Care and Use Committee. Female Fischer rats were selected for their relevance to our ongoing studies on breast cancer therapy.

**Tumor Maintenance and Implantation**

Solid tumors were established via subcutaneous injection of 10\(^6\) CRL-1666 cells in a mixture of 40 \(\mu\)l of serum-free media and 80 \(\mu\)l of Matrigel into the left flank of 3 female Fischer rats. Tumors were allowed to grow to a target volume of 3 cm\(^3\), as measured by calipers in the anterior-posterior, lateral, and cranial-caudal dimensions. When tumors reached the target volume for transfer to the spine of the recipient rat, the flank of the donor rat was shaved and prepared with a povidone-iodine solution. In a sterile environment, the tumor was excised and minced into 5-mm\(^3\) fragments. A recipient female Fischer rat was then shaved and prepared for surgery under sterile conditions, after which a 1-cm incision was made on the lateral flank, and the tumor fragment was then implanted beneath the skin in the subcutaneous space. Finally, the incision was closed with absorbable 5-0 glycolide/lactide copolymer (Polysorb; Tyco Healthcare) running sutures. This process was repeated approximately once every 2 weeks to maintain viable tumor.

**Anesthesia Protocol**

Rats were anesthetized with ketamine/xylazine administered intraperitoneally at a dose of 3 ml/kg body mass. A stock solution of ketamine/xylazine was prepared by combining 25 mg/ml ketamine hydrochloride (Butler Schein Animal Health Supply), 2.5 mg/ml xylazine (Lloyd Pharmaceuticals), and 14.25% ethyl alcohol in buffered 0.9% NaCl solution (Hospira, Inc.). Following administration, the rats were monitored for absence of response to tail pressure to determine degree of sedation.

**Surgical Instruments**

All surgeries were performed under direct observation through a Zeiss operating microscope (ZIIP 84896; Carl Zeiss, Inc.). Spring scissors, Dumont No. 7 forceps, Dumont No. 5 forceps, 10-blade surgical scalpel, cotton-tipped applicators, 5-0 glycolide/lactide copolymer sutures, and surgical autoclips were used. Nanoparticle injections were performed with a 10-\(\mu\)l-gauge Hamilton syringe (Hamilton Co.). All instruments were sterilized by autoclave and placed on sterile drapes throughout the course of the surgery.

**Tumor Implantation**

Tumors were implanted into the L-6 vertebral body (VB) of an anesthetized rat according to methods previ-
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Anesthetized rats were placed on sterile drapes, and the abdomen was shaved and disinfected with a povidone-iodine solution. A 3-cm midline incision centered between the iliac crests was made to the skin, following the procedure described by Mantha et al. to expose the underlying abdominal muscles. A small 2.5-cm superficial incision was made along the midline linea alba of the bilateral rectus abdominus muscles and extended deep until an aperture in the abdominal cavity was visible. The incision was then extended using scissors. The muscle incision extended to the margins of the superior skin incision to permit separate closure of skin and abdominal muscle layers. Consequently, blunt dissection via cotton-tipped applicators was performed with the purpose of mobilizing adipose tissue and bowel to visualize the aorta and vena cava.

The target L-6 VB was identified and drilled by first locating the aortic bifurcation. The deep circumflex iliac artery, which arises approximately 0.3 cm above the bifurcation, was used as a landmark for the L-6 VB. The L-6 VB was then exposed through deep blunt dissection along the left lateral borders of the great vessels and the right lateral border of the left psoas muscle. A high-speed surgical dental drill fitted with a 1-mm bur was used to drill a cavity into the VB for tumor implantation. The cavity was made approximately 0.5 mm superior to the intervertebral disc. Care was taken to protect the great vessels during drilling and exposure of the VB.

Subcutaneous solid flank tumor from each host rat was excised and harvested from carrier animals (see Tumor Maintenance and Implantation) and cut into fragments of approximately 0.1 cm³ for implantation into the 1-mm VB cavity. Following tumor implantation, the drilled cavities were sealed using a dual-cure resin ionomer (Geristore, Den-Mat). The abdominal surgical wound was closed with 5-0 glycolide/lactide copolymer running sutures. The superficial skin incision was closed using surgical autoclips.

Nanoparticle Delivery and Distribution Study Design

Thirty-six female Fischer rats were randomized into control or nanoparticle-injected cohorts. Animals from each cohort were then randomized, 3 to each time point, at 1, 3, 6, 12, 24, and 48 hours. All animals underwent L-6 VB exposure and drilling as described in Tumor Implantation. One flank tumor was harvested for all VB tumor implants. On Day 9 postimplant, all animals underwent an additional transperitoneal exposure of the tumor-bearing lumbar spine for tumor visualization and injection with nanoparticles. Suspensions of starch-coated magnetite (Fe₃O₄) core-shell particles as described above (in Nanoparticle Suspension) were injected to achieve a target intratumor iron concentration of 0.27 mg iron/cm³ of tumor. Tumor volume for dose calculation was measured with calipers in 3 orthogonal directions; anterior-posterior (AP), lateral (L), and cranial caudal (CC). Volume was calculated as length by width by height (AP × L × CC). Nanoparticle suspensions were injected with a 10-μl Hamilton syringe at the circumference of the tumor in both the horizontal and vertical plane to achieve homogeneous particle distribution (Fig. 1). During each injection the needle remained in position for approximately 60 seconds to reduce nanoparticle backflow as the syringe was removed. Animals were then killed at selected time points (1, 3, 6, 12, 24, or 48 hours postinjection) to study particle distribution in relation to time.

Tissue Collection for Analysis

The spine of each animal was harvested immediately following euthanasia by making a longitudinal incision in the skin from the head to the base of the tail. The muscle fascia was then dissected, after which an incision was made at the skull base, and the entire spine was dissected with large scissors. Spines were then fixed in 10% formalin for 12 hours. After fixation, spines were freed of excess tissue and decalcified by a 6-hour immersion in hydrochloric acid. A second dissection was made to localize the tumor-containing VB.

Liver, lungs, spleen, and CSF were harvested to determine the extent of systemic particle distribution. The CSF was harvested with a 23 × 3/8–inch needle inserted into the cisterna magna immediately after euthanasia. Lung, liver, and spleen were collected immediately after euthanasia, weighed, and stored at −80°C until processing for ICP-MS could commence, or they were placed in formalin for histopathological investigation.

Histopathological Investigation

Tissue sections were prepared and stained with H & E. The Perl reaction was used to qualitatively confirm the presence of ferric particles (Fe³⁺) and, if present, assess intratissue distribution. Slides were examined by one pathologist and evaluated for necrosis and pigment.

Segments of tumor were embedded in paraffin, sectioned to a thickness of 10 μm, and stained with H & E to determine extent of invasion by breast adenocarcinoma tumor cells into the osseous body and spinal cord, and with the Perl reaction or PB to analyze nanoparticle distribution (as with other tissues). Nanoparticle distribution in the organs was calculated for 4 randomly chosen high-powered fields within each PB-stained slide of the spinal segments, lung, spleen, and liver. Three slides per tumor were examined. All harvested tissues, with the exception of the spinal segments, were processed using microwave digestion for ICP-MS to measure systemic nanoparticle distribution and concentration.

Mass Spectrometry

Tissues and organs selected for iron quantification were processed for measurement of total iron content by ICP-MS, using methods previously described. Briefly, each tissue sample was microwave-digested in optimal-grade HNO₃ (Fisher Scientific). Scandium (CPI, Inc.) was added as an internal standard to correct instrument drift during analysis, and Seronorm Trace Elements Whole Blood (Sero AS) was used (digested) as an external standard reference material. Blank samples were also digested and analyzed to provide correction. Total iron content of the tissue samples was measured with an Agilent 7500ce ICP-MS device (Agilent Technologies). Each measurement was blank-corrected using the average iron value.
of the reagent blanks, and adjusted based on the recovery of iron from Seronorm. An 8-point calibration curve (0, 1, 5, 10, 50, 100, 500, and 1000 μg/L) was obtained. The analytical limit of detection was calculated by multiplying the SD of the lowest detectable calibration standard (1 μg/L) by 3. For samples with values below the analytical limit of detection, one-half of the limit was substituted.

Thermometry Protocol

Spinal tumors were implanted as described above (in Tumor Implantation), and rectal temperatures were measured in 33 animals studied. Rectal temperatures were measured at 1-second intervals with a fiberoptic temperature sensor (FISO Technologies, Inc.) that was inserted approximately 1 cm into the rectum. Rectal temperatures were measured in all rats as a surrogate measure of core temperature to monitor nonspecific heat stress.21

Twenty-nine female rats bearing L-6 vertebral tumors were randomly divided into 1 of 2 groups on Day 9 postimplantation to receive either nanoparticles (n = 15) or no nanoparticles (n = 14), and then placed in the AMF coil approximately 3 hours after nanoparticle injection. One animal in the nanoparticle cohort died when the rectal temperature reached 44°C and was excluded from further study. For these rats, only rectal temperatures were measured. Rectal temperatures were recorded at 1-second intervals during 20 minutes of AMF exposure and continued for 1 minute after AMF shutdown. MannWhitney t-test (2-tailed, nonparametric t-test) (GraphPad Prism v5.0; GraphPad Software) statistical analysis was performed to compare temperature exposure between the 2 cohorts. A Student t-test was performed for all other statistical analyses using Microsoft Excel. The threshold for statistical significance was set at p ≤ 0.05.

One cohort of 4 randomly selected tumor-bearing rats underwent insertion of a temperature probe into the spinal tumor for intratumor temperature measurements at Day 13. The tumor was exposed by a transperitoneal procedure and was measured to estimate volume for nanoparticle injection. Following nanoparticle injection, a temperature probe was passed through a 14-gauge intravenous catheter and secured with tissue glue to the iliopectineal muscle. The probe was secured with 1 external suture as it coursed externally through the intravenous catheter hub. Day 13 was chosen for the measurement to permit sufficient tumor volume to enable probe insertion. One hour after nanoparticle injection and probe insertion, these animals were exposed to AMF at an amplitude of either 63.3 kA/m for a mean duration of 27.5 minutes (n = 2) or 48 kA/m for a mean duration of 26.0 minutes (n = 2).

For all animals, the abdominal surgical wound was closed with 5-0 glycolide/lactide copolymer running sutures following AMF exposure. The superficial skin incision was closed with surgical autoclips, and at 24 hours the animals were killed, and organs/tissues and spine segments were harvested and prepared for analysis.

Results

Nanoparticle Distribution

Three H & E– and PB-stained sections from each tumor were examined for quantification of nanoparticle distribution at 1, 3, 6, 12, 24, and 48 hours. Nanoparticles could be seen within the tumor at 1 and 3 hours following nanoparticle injection (Fig. 1); however, nanoparticles were undetectable within the tumor by H & E staining and PB staining at 6, 12, 24, and 48 hours after intratumoral injection.
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Injection. Nanoparticles were visible in the bone, specifically in the diapophyses and VB, at 6, 12, and 24 hours.

Nanoparticle distribution within the subdural and intradural space as well as the cauda equina was reviewed. In all nanoparticle-injected animals, there was no evidence of nanoparticle distribution in the cauda equina. However, nanoparticles could be seen in the subarachnoid space of 1 rat at 48 hours (Fig. 1). This finding was not present at 1, 3, 6, 12, or 24 hours in the subarachnoid space of any animal.

Following nanoparticle injection, CSF samples collected from the cisterna magna were submitted to mass spectrometric analysis of iron content. All CSF was collected following euthanasia. Collection of CSF from the cisterna magna was associated with mild trauma to the surrounding tissues and subsequent bleeding. On analysis, CSF samples from nanoparticle-injected animals demonstrated lower iron content at all time points compared with control animals (Fig. 2).

Histological H & E– and PB-stained sections of harvested lungs, liver, and spleen were examined for presence of nanoparticles. Analysis revealed no detectable nanoparticles, either by light microscopy or PB staining, at any of the analyzed time points following nanoparticle injection. Iron concentrations measured by ICP-MS between control and nanoparticle-injected animals revealed statistically insignificant differences (p > 0.05) in either lung or spleen (Fig. 3; Table 1). Control liver samples from non–nanoparticle-injected animals showed a higher absolute iron concentration than nanoparticle-injected animals. The difference was statistically significant at 3, 12, 24, and 48 hours; p values were 0.005, 0.0002, 0.003, and 0.01, respectively.

**Nanoparticle Heating and In Vivo Thermometry**

Amplitude-dependent SLP results are shown in Fig. 4. The BNF particles display relatively poor heating at amplitudes below approximately 30 kA/m, with rapidly increasing heating efficiency between this and approximately 63.3 kA/m, the upper amplitude used in this study. The amplitudes used in this study (48 kA/m and 63.3 kA/m) are denoted by red vertical lines in Fig. 4. Measured SLP at these amplitudes is 242 W/g Fe and .351 W/g Fe, respectively. The study design choice of AMF amplitude for exposing rats with nanoparticles was based on SLP data at both amplitudes to maximize intratumor heating. Based on archival data from studies performed in mice, the upper limits of AMF amplitude were initially selected to be below those expected to cause excessive nonspecific (Joule) heating that arises from eddy currents generated in tissues.21,40

Four rats successfully completed tumor implantation, nanoparticle injection, and AMF treatment at 48 kA/m (n = 2) and 63.3 kA/m (n = 2) with intratumor fiberoptic temperature probe placement, with no systemic hyperthermia and no thermal injury to skin. A schematic diagram depicting the experimental protocol can be seen in Fig. 5. The mean peak intratumor temperature is the highest recorded temperature during the AMF exposure.

![Fig. 2](image)

**Fig. 2.** A: Chart showing systemic particle distribution as assessed by ICP-MS versus time. Data represent the average iron concentration per milliliter CSF for 3 samples at each time point. B: Schematic drawings depicting experimental design. Following intratumor injection of nanoparticles, animals were killed and CSF was collected from the cisterna magna.

![Fig. 3](image)

**Fig. 3.** A: Chart showing lymphoreticular iron concentration. Graph of spleen, liver, and lung iron concentration for control versus nanoparticle (NP)-injected animals. All data are plotted as an average of the concentration at a given time point. B: Schematic drawings depicting experimental design. Following intratumor injection, animals were killed at set time points, and lung, liver, and spleen were collected for histopathological investigation and ICP-MS.
For animals treated with 63.3 kA/m, average rectal and tumor temperatures were 35.3°C and 43.2°C, respectively, with a time to peak temperature of 24.3 minutes. For animals treated with 48 kA/m, average rectal and tumor temperatures were 35.7°C and 40.6°C, respectively, with a time to peak temperature of 24.6 minutes.

In contrast to the intratumor thermometry study, the first animal of the experimental rectal thermometry cohort treated at 63.3 kA/m quickly reached a rectal temperature of 44°C; thus subsequent animals (n = 28) were treated at 48 kA/m for 20 minutes at 3 hours following injection of nanoparticles. A schematic diagram depicting the experimental protocol can be seen in Fig. 6.

For animals treated at 48 kA/m for 20 minutes, there was no statistically significant difference (p = 0.44) in mean final rectal temperature when nanoparticle-injected animals were treated in the AMF coil (36.2°C ± 1.4°C) versus those that received no nanoparticles (36.7°C ± 1.6°C) (Fig. 6).

Spinal Cord Histopathological Findings and Functional Analysis

Four animals exposed to AMF heating with nanoparticle injection were examined for histopathological evidence of necrosis in the cauda equina at 24 hours after treatment. No evidence of necrosis within the cauda equina was apparent on examination of H & E–stained sections. A pathologist specializing in osseous tumors examined the H & E–stained samples obtained in the nanoparticle-injected, AMF-treated, intratumor thermometry cohort (n = 4). Although 25%–75% tumor necrosis was evident within the treated tumors, a causal link for necrosis with nanoparticle hyperthermia was not established.

Thirty-two animals, including rectal thermometry (n = 28) and intratumoral thermometry (n = 4) cohorts, were used in the pre- and postoperative functional analysis. Preoperatively all animals were neurologically intact, with a Basso-Beattie-Bresnahan score of 21. At 24 hours posttreatment, all 32 animals maintained a Basso-Beattie-Bresnahan score of 21. There were no deaths associated with intratumoral nanoparticle injection or with AMF treatment at 48 kA/m.

Discussion

Ferromagnetic susceptor nanoparticles generate heat when exposed to an AMF and have been documented to produce neoplastic tissue thermoablation. The goal of this study was to develop and characterize a nanoparticle delivery protocol that would permit intratumoral distribution while avoiding significant systemic distribution, thereby allowing for selective heating of tumor tissues. This was performed for the first time in an intraosseous rat model of metastatic spine disease. This model has been previously used to test numerous treatment modalities ranging from local chemotherapy to radiotherapy. In the context of the current study, this model tests the safety of applying local hyperthermia to tumors abutting the highly sensitive neural tissues of the spine and great vessels.

The results of our study demonstrate that fractionated circumferential nanoparticle delivery provides a method for achieving diffuse intratumoral nanoparticle distribution. Time-dependent analysis of nanoparticle distribution demonstrates that particles maintain a consistent distribution within the tumor mass at 1 and 3 hours after injection, with migration into the surrounding bone at 6,

### TABLE 1: Comparison of control and nanoparticle-injected rats for tissue iron concentration in lymphoreticular organs*

<table>
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<th>Organ</th>
<th>Hours Postinjection</th>
<th>p Value</th>
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*All data reflect results of ICP-MS measurements. There was no statistically significant difference in iron concentration as measured by ICP-MS for spleen or lung tissue at any time point measured.
†There was a statistically significant difference in iron concentration in liver tissue for control versus nanoparticle-injected animals at 3, 12, 24, and 48 hours.

![Fig. 4. Chart showing measured BNF particle SLP by using methods described in the text. Vertical lines indicate magnetic field amplitudes used in the study.](image-url)
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Figure 5. Intratumor and rectal temperatures for nanoparticle-injected and coil-treated animals. A: Peak rectal and intratumor temperatures for individual rats. B: Average rectal and intratumor temperatures and time to peak temperature. C: Schematic drawings depicting experimental design. Animals were treated with intratumor injection of nanoparticles, and then approximately 1 hour later they were placed into the AMF for rectal and intratumoral thermometry with fiberoptic probes.

12, and 24 hours. Furthermore, at 48 hours nanoparticles could be seen in the subdural space, presumably within macrophages recruited to the area. This finding suggests that for optimal thermal therapy, magnetic nanoparticle hyperthermia may be most efficacious within 3 hours of nanoparticle injection because that was when the highest intratumor concentration of particles was observed. Furthermore, treatment with AMF coil therapy may be unsafe 48 hours after injection of nanoparticles, because particle accumulation in the subarachnoid space and subsequent heating may cause severe neurological damage.

Iron concentration in lung, liver, spleen, or CSF was not significantly increased in nanoparticle-treated animals up to 48 hours after nanoparticle injection. The lung, liver, and spleen are organs of the lymphoreticular system and thus involved in iron metabolism. Macrophages routinely ingest iron in response to inflammatory states, and previous studies have demonstrated that superparamagnetic iron in the systemic distribution is sequestered within macrophages of the lymphoreticular system. Histopathological examination of tissues failed to establish the presence of nanoparticle-laden macrophages in lung, liver, or spleen. Furthermore, iron quantification demonstrated tissue iron concentrations within the normal range for organs of the lymphoreticular system and in the CSF. Taken together, these findings suggest no significant accumulation of nanoparticles or nanoparticle-laden macrophages in peripheral organs. Notably, control animals demonstrated statistically significantly increased iron at several postoperative time points. Causes of this may include operative variables, such as trauma to the collected tissues and bleeding associated with the cisterna magna collection. Further research into the cause of this abnormality may be warranted in future ICP-MS studies of systemic ferromagnetic nanoparticle distribution, if future therapy studies reveal significant effects from the procedure. Additionally, use of a noniron nanoparticle or a particle with a labeled coating would enhance iron studies to trace systemic distribution.

We acknowledge that there are limitations to the methods of detection used in this study and that it is possible that iron particles became systemically diluted to concentrations falling within the normal variability of endogenous iron. The ICP-MS methods are sufficiently quantitative, with established resolution to 1 part in approximately 10^9, provided sample preparation and calibration with appropriate standards are established. Individual variations of iron content, however, can be significantly greater than this (from studies with mice), leading to an inability to resolve slight elevations in iron content. On the other hand, if accumulating nanoparticles in the periphery are below the limits of clinical detection, then they are unlikely to have a clinically relevant impact in patients who receive nanoparticle therapy. This finding suggests that nanoparticle concentration in the systemic distribution remains extremely low following intratumoral injection and is unlikely to produce clinically relevant effects in organs after the introduction of the AMF.

Thermometry demonstrated that AMF treatment following nanoparticle injection generates higher intratumoral temperatures, while rectal temperatures remain within normal limits. Although statistically not sufficient (limited by sample size), the rats exposed to 63.3 kA/m reached higher intratumoral temperatures than the 48 kA/m group, consistent with previous experiments demonstrating that heat generated and deposited by the
nanoparticles depends upon the intensity of the AMF. The observation that rectal temperatures for the 63.3 kA/m cohort remained similar to those in rats exposed to 48 kA/m in the initial thermometry study is noteworthy. This suggests that future efficacy studies include a cohort to be exposed 63.3 kA/m with minimal risk.

In the subsequent rectal thermometry study, however, 48 kA/m was chosen because the first animal exposed to 63.3 kA/m died of elevated internal temperature. One explanation for this observation is the size of the tumor. Although animals were treated within 13 days of tumor implantation, there is some variation of tumor volume, leading to different susceptibility profiles. Furthermore, proximity of the tumor mass to the abdomen or great vessels and potential growth of the tumor into the vascular bundle may influence elevations in rectal and core temperatures. Future experiments are needed to explore limits of AMF exposure and therapy conditions for large tumors to avoid disrupting the cortical bone of the VB and to avoid extension into the perivascular tissues of the aorta or vena cava. Preclinical imaging using small-animal MRI and CT scanning may enable preoperative screening to identify appropriate combinations of nanoparticle and AMF therapy for each subject.

This is the first study demonstrating specific tumor heating in a rat model of metastatic spine disease by using magnetic nanoparticles, and the limitations of the study must be noted. The current work was a feasibility study to determine whether this interventional approach has merit, with potential for future development. The hypothesis to be tested in the current study design is that magnetic nanoparticles can be injected into spinal tumors and exposed to an AMF that will potentially deposit heat while avoiding acute toxicity in spinal tumors in a rat model. Hence, intratumoral thermometry was included to provide a proof-of-concept adjunct with preliminary data to support further study and development of the potential heating for therapy. In the current study, we have demonstrated that it is possible to measure intratumoral temperature during heating, and we have also tested the concept that this therapy has the potential of achieving hyperthermic intratumoral temperatures without significantly impacting systemic temperature. Nonetheless, the sample size of the intratumoral thermometry arm was small (n = 4). This not only limits the conclusions that can be drawn from this arm, but as stated in the foregoing discussion, also prohibits the statistical evaluation of intratumoral temperatures achieved per field dose (63.3 kA/m vs 48 kA/m). These aspects require additional study with an experimental design that is focused on such end points.

Additional limitations of the study include varied tumor sizes, narrowed tissue sampling, and an absence of functional and survival analysis. Uniform tumor size may eliminate variability in response to AMF levels, and pre-intervention imaging will be helpful to standardize tumor size across experimental and control groups. Tissue sampling and analysis for tumor necrosis at 24, 48, 72, and 96 hours posttreatment will be necessary to fully characterize whether and how thermal therapy affects tumor biology or induces tumor necrosis. Viability staining may enable analysis of the biological response in surrounding tissues following locally (intratumorally) elevated temperatures. Data addressing long-term functional outcomes and survival of animals treated with nanoparticle and AMF therapy are also necessary for understanding the clinical potential of this technology. These and other considerations are the focus of ongoing experiments to further develop this technology and assess its applicability for clinical use.
Nanoparticle hyperthermia for spine tumors

Conclusions

Nanoparticle-mediated thermal therapy by AMF heating at 48 kA/m induces sufficient specific heat to increase intratumor temperature to 41°C without raising rectal temperature. In our experimental cohort, 24 hours after AMF treatment no functional impairment and no evidence of necrosis within the cauda equina on H & E staining were observed. Furthermore, no evidence of significant nanoparticle accumulation in the CSF or lymphoreticular organs at any time following nanoparticle injection was observed. These observations support the hypothesis that nanoparticle-mediated hyperthermia can be a safe therapeutic tool to treat tumors of the spinal column.

Disclosure

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