ORIGINAL REPORT

Magnetic resonance imaging and spectrometric study of the distribution of thermotherapeutic magnetofluid after intra-arterial administration in an experimental model of liver metastases


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KEYWORDS
Liver metastases; Neoplasm; Experimental; Animal experimentation; Nanoparticles; Iron concentration

Abstract

Objective: To use imaging and laboratory techniques to evaluate the vascular distribution of magnetofluid in a rat model of liver metastases.

Material and methods: The livers of 33 WAG/Rij Crl rats were seeded with CC-531 colorectal cancer cells. After we checked tumor development, 10 rats received hepatic intra-arterial infusions of Lipiodol® with nanoparticles of Fe3O4 in suspension, and 5 were reserved as controls. Axial STIR (TR: 3600 ms/TE: 29 ms/TI: 130 ms) and gradient-echo (GE) (120/4 and 120/14) MRI sequences were acquired on a 1.5 T scanner. After necropsy, rats were classified into two stages according to tumor development: early (<10 metastases, each <3 mm) or advanced (>10 metastases, each >3 mm). Samples of liver and of metastases were taken from the 15 animals for quantification of iron concentrations by inductively coupled plasma mass spectrometry (ICPMS). The data were analyzed using nonparametric tests; values of $p<0.05$ were considered significant.

Results: Five animals had early tumor development and five had advanced tumor development. In the GE sequences, early stage metastases showed homogeneous signal reduction attributable to the presence of magnetofluid. Spectrometry found significant differences between the iron concentration in rats with early stage metastases and controls ($p=0.002$) as well as between rats with early stage metastases and those with late stage metastases ($p=0.001$). The ratio of exogenous iron in metastases and in liver in early stage rats was 2.6:1. The concentration of exogenous iron in the liver was significantly different from that in tumors only in early stage animals ($p=0.043$).


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Palabras clave
Metástasis hepáticas; Neoplasia; Experimental; Experimentación animal; Nanopartículas; Concentración de hierro

Administración intrarterial de un magnetofluido termoterapéutico en un modelo experimental de metástasis hepáticas. Estudio de distribución con resonancia magnética y espectrometría

Resumen
Objetivo: Valorar la distribución vascular de un magnetofluido por técnicas de imagen y laboratorio, en un modelo de metástasis hepáticas.

Material y métodos: El hígado de 33 ratas WAG/RijCrl fue diseminado con células de carcinoma colorrectal CC-531. Tras comprobar desarrollo tumoral, diez ratas recibieron infusionsintrarteriales hepáticas de Lipiodol\textsuperscript{8} con nanopartículas de Fe3O4 en suspensión, y cinco se reservaron como controles. Posteriormente, en RM de 1,5 T se practicaron secuencias axiales STIR (TR: 3.600 ms/TE: 29 ms/TI: 130 ms) y eco de gradiente (EG) (120/4 y 120/14). Tras necropsia, según desarrollo tumoral, las ratas se clasificaron en dos estadios: precoz (< 10 metástasis, de < 3 mm), avanzado (> 10 metástasis, de > 3 mm). De los 15 animales se tomaron muestras de hígado y metástasis, para cuantificar mediante espectrometría (ICP-MS) las concentraciones de hierro. En el análisis estadístico se emplearon pruebas no paramétricas. Se consideraron significativos valores de p < 0,05.

Resultados: Cinco animales presentaron afectación precoz y cinco, avanzada. En secuencias EG, las metástasis en estadio precoz mostraron disminución homogénea de señal atribuible a presencia de magnetofluido. La espectrometría demostró diferencias significativas entre la concentración de hierro determinado en metástasis de ratas en estadio precoz y control (p = 0,002), y entre animales en estadio precoz y avanzado (p = 0,001). La razón entre hierro exógeno metastásico y hepático en ratas en estadio precoz fue 2,6:1. La concentración de hierro exógeno hepático y tumoral mostró diferencias significativas sólo en animales en estadio precoz (p = 0,043).

Conclusiones: RM y Espectrometría permitieron evaluar la distribución vascular hepática del magnetofluido, y revelaron su desigual afinidad por metástasis en diferentes estadios.

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Introducción

Colorectal cancer is one of the most common neoplasms in the Western world, and it accounts for a high rate of liver metastatic disease. At present, the treatment of choice for liver metastases is surgical resection, since it shows the best long-term survival, with five-year survival rates of around 24–38%\textsuperscript{1} but they can reach 58% in series of selected patients.\textsuperscript{2} However, due to the location of tumor implants or the clinical condition of patients, only 8–27% of patients with liver metastases will be candidates for surgical resection.\textsuperscript{3}

For patients who are not suitable candidates for surgery, alternative local therapies have been developed such as transparietal percutaneous ablation techniques (radiofrequency, thermoablation and ethanol injection), or transarterial embolization procedures, chemoembolization or radioembolization. However, the uneven success that these non-surgical techniques show in general\textsuperscript{4–7} makes it necessary to research for new alternatives.

Magnetic nanoparticles, when exposed to an external magnetic field, can produce an energy transfer inducing hyperthermia. Their clinical use has been tested in different types of tumors, especially in prostate cancer.\textsuperscript{8} A feasible therapeutic option in the hepatic field would be the administration of intra-arterial magnetic nanoparticle suspensions, so that they remain deposited in the tumor tissue. In this location, and following exposure to an external magnetic field, the suspension or magnetofluid would cause the thermal ablation of the neoplastic tissue.

With the aim of being used in thermotherapy of liver metastases, we have developed a magnetic fluid based on a suspension of iron oxide nanoparticles in iodized oil Lipiodol\textsuperscript{8} (Guerbet Laboratory, France), a substance to which the tumor tissue shows great affinity.\textsuperscript{9} As a preliminary step and prior to assessing the thermosterapeutic ability of the magnetofluid, we have evaluated, using MRI and spectrometry, its vascular distribution following intra-arterial infusion in a rat model of multiple liver metastases. This way, through qualitative (MRI) and quantitative (spectrometry) estimation of the presence and concentration of iron in the different hepatic tissue (healthy and metastatic), it is intended to estimate the diffusion of the magnetofluid on the hepatic arterial bed, and also determine its higher or lower affinity for the neoplastic tissue.
Material and methods

Development of the experimental model (Fig. 1)

The experimental study was performed on an initial number of 33 male rats WAG/RijCrl weighing 199–316 g. All procedures complied with the current animal testing regulations established by the Spanish Government (Royal Decree 1201/2005, October 10).

Rats were kept in an animal facility under standard conditions: 22 °C average temperature, 55% relative humidity, 12-h periods of alternating light and darkness, and received standard feed and water ad libitum.

On day zero of the test, animals were inoculated with syngeneic cells of colon adenocarcinoma (cellular line CC-531), in order to develop liver metastases according to the splenic reservoir technique. With this procedure, induction of visual perceptible metastases is achieved from week four after inoculation in more than 70% of the animals.10 The inoculation procedure required surgical exposure of the spleen through a subxiphoid midline laparotomy, to later perform a direct intra-splenic injection of 250,000 tumor cells suspended in 0.5 ml of Hank’s solution. Five minutes after the injection, splenectomy was performed to avoid the development of a primary tumor.

Thirty days after tumor inoculation, intra-arterial infusion of magnetofluid was performed. During surgical performance, it was found that 21 rats had developed metastases. Procedures were performed in sequence, and the animals were laparatomized randomly. Rats with no tumor involvement were discarded and sacrificed. Ten rats were appropriately infused and six died during infusion processes. The remaining five were not infused with suspensions and were reserved as controls.

Anesthesia

During the process of tumor inoculation, an inhalation induction with halothane was used. During the magnetic fluid infusion and imaging tests, intraperitoneal anesthesia was used with an initial injection of diazepam (5 mg/kg) followed by ketamine (100 mg/kg) 10 min later.

Magnetic fluid

The magnetofluid was produced from Fe3O4 magnetic nanoparticles ranging from 3.8 to 7.1 nm. During the synthesis process, nanoparticles were capped by lipid organic ligands, especially oleic acid, that through ultrasonification would allow the suspension in the iodized oil Lipiodol®. This way, suspensions of 2 mg of iron oxide nanoparticles in 0.2 ml of Lipiodol® were prepared, to be later individually infused in each animal.
Infusion procedure and imaging techniques

The surgical procedure of magnetofluid infusion in the ten selected animals required a midline laparotomy to enable exposing the visceral arterial vessels following liver and stomach retraction. By using a binocular surgical microscope (Leika M651) and following identification of the celiac trunk, a reversible ligation of the main branches was performed, except for the hepatic artery, with the aim of driving toward this artery most of the celiac vascular flow. Subsequently, a direct puncture of the celiac trunk was performed using a 30 G needle connected to an elongated catheter and an infusion pump pre-charged with magnetic fluids (Fig. 2). Once the device was in place, slow infusion of the suspension (3 min) was performed. The device was withdrawn and reversible ligation of the celiac artery and vigorous compression for hemostasis was performed. Finally, after removing the vascular occlusion, the laparotomy was closed.

Within the first 12 h following the administration of suspensions, the animals underwent MRI examinations on a 1.5 T Siemens Symphony scanner. A standard head coil was used and axial STIR (TR: 3600 ms; TE: 20 ms; TI: 130 ms [3600/20/130]; section thickness: 3 mm; matrix: 268 × 512) and GE (120/4 [GE-DP] and 120/14 [GE-T2]); flip angle: 20°; section thickness: 3 mm; matrix: 312 × 512 [GE-DP] or 268 × 512 [GE-T2]) sequences were obtained. On STIR sequences, metastases appeared as well-defined hyperintense masses. On GE sequences, the presence of magnetic nanoparticles led to a drop in signal intensity (SI) wherever they were located, both in healthy liver and tumor lesions, and this finding would corroborate appropriate vascular diffusion of the suspensions. Given the size of the animals used and the tumor implants that were hypothetically to be developed in livers, MR imaging would not enable appropriate spatial location of metastases <3 mm, especially in GE sequences. This contingency would prevent an accurate quantification of iron concentration using MR imaging in metastatic tissue of minimum size, reason for which this diagnostic option was not considered.

Assessment of the neoplastic induction and iron determination

Immediately after the imaging studies, both injected and control rats were euthanized by deep anesthesia and cervical dislocation. Livers were extracted and the number and size of metastases were determined by visual analysis. Two groups of tumor growth were considered. On the one hand, early stage neoplastic infiltrations corresponding to non-confluent lesions (separated by healthy parenchyma) <3 mm, and with no more than ten visually identifiable lesions were considered. On the other hand, livers with extensive neoplastic infiltrations in advanced stage were characterized by having metastases >3 mm, or more than ten visually detectable lesions.

Afterwards, samples of liver and neoplastic tissue were taken in order to determine iron concentrations. Liver samples consisted of various cores taken from different locations with a total weight of at least 100 mg. Similarly, metastatic tissue samples consisted of material from various neoplastic lesions. Healthy liver tissue was carefully removed from tumor samples in order to obtain completely enucleated metastatic lesions. Once identified, specimens were frozen dry to −20 °C until processing. For iron quantification, samples underwent an acid digestion process, and the resulting solutions were analyzed by spectrometry (Inductively Coupled Plasma-Mass Spectrometry). During this process, the average iron concentration in micrograms (µg) per gram of tissue was determined.

Under normal conditions, there is a certain quantity of intrinsic hepatic iron mainly located in the hepatocytes and in blood hemoglobin. Given its chemical nature, this iron does not have the ability to induce heat but can be detected by spectrometry and is indistinguishable from exogenous iron. With the aim to quantify baseline iron concentrations, samples from the control group were taken. Mean values of iron concentration from the control group would be subtracted from the specific values obtain from the samples of fluid-infused animals. This way, the quantity of iron present in healthy and metastatic tissue attributable to exogenous administration could be estimated.

Statistical analysis

The mean, standard deviation (SD), median (Md) and range (maximum—minimum), as well as a box plot were used for determination of quantitative variables relating to iron concentration. Statistical analysis was performed with non-parametric tests (Wilcoxon test for related samples). Once significant differences between groups had been demonstrated using the Kruskal–Wallis test, comparison between possible pairs of groups was performed a posteriori using the Mann–Whitney U-test. Values p < 0.05 were considered statistically significant.

Results

Of the ten animals in which surgical procedures and magnetic fluid infusion were successfully completed, five showed early stage metastatic growth, while the other five presented advanced stage metastases. In the control group, three rats showed early stage metastatic growth and two showed advanced stage metastatic growth.

STIR sequences demonstrated hyperintense tumor lesions in all animals, that is, in fluid infused and control animals.
GE sequences performed on the control group showed livers with signal characteristics similar to those in the paraspinal musculature, with drop in SI that was more conspicuous in GE T2-weighted sequences. Moreover, on these sequences with longer TE, it was possible to recognize metastases detected on STIR sequences, since tumor tissue showed lower signal drop than healthy liver tissue. On the other hand, GE-DP sequences, and especially GE-T2 performed on infused animals, showed an acute decrease in hepatic SI attributable to the presence of magnetic nanoparticles in the organ vascular tree (Fig. 3). In animals at early stages, the signal drop in GE-PD and EG-T2 weighted sequences involved the entire liver and tumor tissue could not be distinguished from healthy liver tissue (Fig. 4), and therefore, selective SI measurements on the metastases could not be performed. However, in animals at advanced stages, tumor tissue showed a more heterogeneous aspect, with areas of SI similar to those of the paraspinal musculature, clearly distinguishable from the hypointense healthy liver, alternating with areas of intense signal drop. These findings suggested the imaging manifestation of a more irregular vascular distribution of magnetofluids (Fig. 5).

Following imaging studies, samples of liver tissue and metastases were taken from each animal to quantify the iron concentration by spectrometry. Endogenous iron concentration levels were determined from samples taken from the controls. Samples were taken from infused rats to determine iron concentration corresponding to both endogenous and exogenous iron (from nanoparticles). Iron concentrations in both groups of animals are shown in Table 1 and Fig. 6.

The statistical analysis of results demonstrated significant differences in iron concentration between liver and metastatic tissue (Kruskal–Wallis: \( p = 0.037 \) and \( p = 0.02 \), respectively). A detailed analysis showed significant differences in hepatic iron between the early stage and the control groups (\( p = 0.017 \)), but no differences were found between advanced and control groups (\( p = 0.177 \)). Regarding tumor metal, there were significant differences between values determined in rats with metastases at early stages and control groups (\( p = 0.002 \)), and between rats at early and advanced stages (\( p = 0.001 \)), but no differences were found between those rats at advanced stages and controls (\( p = 0.228 \)).

After subtracting the mean values of endogenous iron found in the control group, from iron values found in fluid-infused animals, the mean concentration attributable to exogenous administration in the early stage group was \( 172.2 \, \mu g/g \) in tumor tissue and \( 65.2 \, \mu g/g \) in liver tissue. In these animals, the ratio between the concentration of exogenous iron present in neoplastic tissue and healthy tissue was 2.6:1, that is, tumor tissue accumulated 2.6 times more iron than healthy liver tissue. On the other hand, mean value of exogenous iron determined in rats at advanced stages was \( 22.7 \, \mu g/g \) in metastatic tissue and \( 43.1 \, \mu g/g \) in healthy tissue. In this group, the ratio between exogenous iron in neoplastic tissue and healthy liver tissue was 0.5:1, that is, advanced metastases accumulated half as much exogenous iron than healthy liver tissue.

Finally, the intragroup comparative study between exogenous iron concentrations in liver and metastases showed significant differences only in the early stage group (\( p = 0.043 \)).

**Discussion**

Colorectal cancer is one of the most common neoplasms in the Western world. It is also a disease with a marked tendency to cause metastatic liver dissemination, fact that occurs in almost half the patients during the course of the disease, and even in 15–25% of the cases metastases are already present at diagnosis.\(^{14,15}\)

The treatment of choice in liver metastases is surgical resection but this is not viable in more than two thirds of the cases.\(^{3}\) For these patients, various local alternatives have been developed but unfortunately with limited therapeutic efficacy. In this respect, thermoablation with radiofrequency provides five-year survival rates of around 30–40\%.\(^{4,5}\) Transarterial procedures, such as chemoembolization, can cause complications in up to 4.4% of patients,\(^{6}\) also providing variable therapeutic outcomes and survival rates, depending on the selection criteria and the combination of chemotherapeutic agents used.\(^7\) There is therefore a clear need for developing new therapies, especially for patients with multifocal metastases.

Despite its limitations, given its low systemic toxicity, theromtherapy presents an important advantage in antitumor therapy. This has led to study the possible application
of metal nanoparticles capable of generating hyperthermia for the local treatment of neoplastic disease. By using direct administration to the tumor bed, magnetic nanoparticles have been used in experimental models of liver and breast cancer,\textsuperscript{13,16} and at a clinical level, in various types of recurrent neoplasms.\textsuperscript{8,17} However, at an experimental level, transarterial administration has provided a more homogenous distribution of nanoparticles in neoplastic tissues, ensuring a more effective therapeutic hyperthermia.\textsuperscript{13}

The aim of all liver transarterial procedures is to reach the neoplasm through its arterial bed. This is based on the fact that the healthy liver gets its blood supply from the venous system, whereas metastases and micrometastases are supplied by the hepatic artery.\textsuperscript{13,18} In our experimental

\begin{itemize}
  \item [\textbf{Figure 4}] Rat with tumor growth at early stage. Various subcapsular metastases are identified (yellow circle) on the liver surface. Following magnetofluid administration, STIR images show a hyperintense and well-defined neoplastic mass (yellow circle). On GE-T2 images, the mass cannot be distinguished from the healthy liver tissue and both show a marked drop of SI secondary to the presence of iron oxide nanoparticles in the vascular tree.
\end{itemize}

\begin{itemize}
  \item [\textbf{Figure 5}] Animal at advanced stage of tumor infiltration. Profuse neoplastic growth of the medial lobe (arrows) is observed. Following magnetofluid infusion, STIR images show hyperintense tumor masses (arrows) within the liver parenchyma. On GE-PD images, neoplastic lesions (arrows) show heterogenous foci of signal drop. This finding seems to be caused by an uneven vascular distribution of the nanoparticles within the tumor tissue.
\end{itemize}
In the experimental model, the tumor bed would be reached via intra-arterial administration of a fluid consisting of iron oxide nanoparticles in Lipiodol®. Given the affinity of the tumor tissue for this iodized oil, the magnetofluid would mainly be contained in the neoplastic bed.

Intratumor vascular distribution of the magnetofluid has been studied in models of liver neoplasms induced by direct implant of tumor cells. Our work has been developed on a different model of multiple metastases, generated by hematogenous spread. Since the achievement of a selective deposit of nanoparticles in tumor tissue will avoid lesions in the healthy liver parenchyma, assessing nanoparticle distribution is of crucial importance before determining its potential ability for thermal induction.

The GE sequences used for quantification of liver iron overload in humans, very sensitive to paramagnetic substances, were of great use in our experimental model. On the one hand, in animals from the control group these sequences allowed individualization of metastases in relation to the healthy liver. Indeed, as spectrometry showed, the mean concentration of endogenous iron found in non-infused animals was higher than the concentration in tumor tissue, allowing identification of metastases on GE-T2 weighted sequences, since they showed lower signal drop than the liver. The use of GE sequences in infused animals provided an initial evaluation of the intra-arterial distribution of the magnetofluid, since a significant signal drop in the organ vascular bed attributable to the presence of nanoparticles was observed. Regarding neoplastic lesions, failure to distinguish tumor tissue from healthy tissue in infused animals in early stages suggested an appropriate distribution of magnetofluid in the tumor tissue, probably because small metastases had a preserved vascular bed. However, failure to distinguish between healthy liver tissue and tumor tissue prevented performing iron quantifications based on changes in SI. On the other hand, in animals with advanced metastatic disease, areas with no drop in SI, that is, free of iron deposits, were also observed in areas with extensive neoplastic infiltration. This finding posed the suspicion that the arterial vasculature in these voluminous neoplastic tissues was compromised.

Spectrometry also allowed evaluation of the distribution of nanoparticles by quantification of iron in different tissues. In the analysis of results obtained from healthy liver samples, significant differences in iron concentration were found between animals at early stages and controls, but not between early and advanced stages. This suggests an appropriate hepatic distribution of the fluid in infused rats. Regarding the study of tumor tissue, significant differences were found between the quantities of iron present in early metastases and in the other groups, but it was not so between the iron found in animals with early stage metastases and controls. These data showed that only early metastases accumulated a substantial quantity of magnetic fluid.

The presence of endogenous physiologic iron was also considered in the evaluation of spectrometric analyses. In this sense, results obtained when estimating iron concentrations following subtraction of endogenous iron from rats with early and advanced metastases highlighted the differences between the quantities of iron in tumor tissue in both groups of animals. Additionally, it was also confirmed a significant difference between the quantity of exogenous iron in tumor tissue and healthy tissue in rats with early stage metastases was also confirmed. The importance of this finding lies in the fact that this exogenous metal will have the ability to induce hyperthermia, and the achievement of high concentration of magnetic nanoparticles in tumor tissue versus healthy tissue will prevent the latter from being damaged by heat.
The possibility to compare our results obtained with results obtained in other experimental models is complicated given the wide variability of iron concentrations observed in the different animal species under study. However, a useful comparative method to evaluate selectivity of tumor tissue in different models is based on establishing the ratio between iron concentration in metastatic tissue and healthy tissue once the endogenous iron is subtracted. Published studies have found exogenous iron concentrations in tumor tissue between 3.5 and 5.3 times higher than those found in healthy liver tissue. When analyzing our model, two different results were observed. On the one hand, in rats with early stage metastases a considerable deposit of iron nanoparticles in tumor tissue was achieved, in a concentration 2.6 times higher than in healthy tissue. Although this value proved to be lower than values reported by other models, differences in iron concentration were evident and agreed with previously published papers. However, in rats with advanced stage metastases, the analysis of iron concentration in enucleated metastases showed a limited deposit of iron in comparison to healthy liver tissue. It can be concluded from our study that magnetic fluid deposits did not show a linear increase with the progressive increase of tumor mass, perhaps also because of the associated necrosis. These results were consistent with the observation on MRI of areas with preserved SI in GE sequences, within the neoplastic tissue, suggesting the presence of perfusion defects in areas of marked tumor infiltration.

In conclusion, despite the limitations of our study given by the low number of animals included in each group and the MRI technology used, rather clinical than specifically experimental, two interesting contributions drawn from this work will be useful in future research studies. First, contrary to other experimental models, our study has been developed using a hematogenous spread model, more similar to the natural process of metastatic spread, and it has demonstrated its usefulness in new therapeutic trials. Second, in light of the results obtained, the possibility that there might be substantial differences in perfusion in neoplastic lesions of similar type but at different stages must be considered in experimental studies on new hypothetically useful in vitro therapies, since this aspect might determine their success to a greater or lesser extent.

Conflict of interest

The authors declare not having any conflict of interest.

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