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Application of luteinizing hormone-releasing hormone–ferrosoferric oxide nanoparticles in targeted imaging of breast tumors

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Abstract

Background: Ferrosoferric oxide (Fe_3O_4) nanoparticles are a commonly used magnetic resonance imaging (MRI) reagent. Luteinizing hormone-releasing hormone (LHRH) is highly expressed on the surfaces of tumors, but its expression is low or absent in the corresponding normal tissues, allowing it to be used for targeted imaging and treatment.

Methods: We prepared Fe_3O_4 nanoparticles using a chemical co-precipitation method, performed coupling with chitosan to prepare LHRH-Fe_3O_4 nanoparticles, and explored the application value of LHRH-Fe_3O_4 nanoparticles in targeted imaging and treatment of breast tumors through *in vitro* and *in vivo* experiments.

Results: The particle size of the LHRH-Fe_3O_4 nanoparticles was 10 nm, and they could be taken in by human MCF-7 breast cancer cells. The nanomaterial had low cytotoxicity. *In vivo* MRI experiments showed that LHRH-Fe_3O_4 could effectively concentrate on the tumor under the action of a magnetic field. It also had a good negative enhancement effect that significantly reduced the signal intensity of the T_2 field, allowing it to be used as a contrast agent of the T_2 field.

Conclusion: LHRH-Fe_3O_4 nanoparticles serve the purpose of targeting contrast agents to target sites and are expected to be used for targeted imaging and treatment of cancers with high LHRH expression.

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Ferrosoferric oxide, luteinizing hormone-releasing hormone, magnetic resonance imaging, breast tumor, nanoparticles, targeted imaging

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Introduction

Malignant tumors are a serious threat to human life and health. Their incidence has been increasing on an annual basis and their mortality is surpassing that of cardiovascular and cerebrovascular diseases, making them the leading threat to human health. Molecular imaging based on molecular biology, imaging science, and other subjects is expected to reveal tiny tumor lesions at the molecular level, facilitating early diagnosis of tumors. However, although magnetic resonance imaging (MRI) is a major molecular imaging examination, contrast agents are susceptible to inducing allergic reactions and are phagocytosed by macrophages; additionally, their surfaces are not easily modified, potentially resulting in insufficient targeting. Thus, the application of contrast agents affects the MRI-based diagnosis of tumors. A main focus in the field of MRI is the development of highly specific targeted contrast agents to increase the detection rate of tiny tumor lesions. Ideal targeted contrast agents should have the following characteristics[1–3]: simple preparation, low cost, a small particle size that allows easy access to target areas, high specificity and affinity for receptors, no toxic adverse effects, and the ability to reach an adequate concentration in the target area to obtain a good imaging effect.

Ferrosoferric oxide (Fe_3O_4) nanoparticles are positively charged. Their magnetic property is relatively strong, and their preparation is relatively simple. After they enter the body, they can be recognized by immunoglobulins in the blood and then phagocytosed by the reticuloendothelial system. Finally, they are excreted through the kidneys and liver. Compared with other MRI reagents, Fe_3O_4 nanoparticles are excellent MRI T_2 contrast agents[5,6] because of their good biocompatibility and magnetic resonance signal sensitivity.[4] As early as 1996, the US Food and Drug Administration had already approved ferumoxsil and ferumoxide, two magnetic iron oxide nanomaterial drugs. These nanoparticles are easily agglomerated within the body and cleared by the reticuloendothelial system because of their surface hydrophobicity and large surface ratio. Therefore, the surfaces of these nanoparticles must be modified to increase their hydrophilicity and prolong their circulation half-life. Polysaccharides such as chitosan and heparin are used as surface modification materials and can increase the entrapment efficiency and load capacity of the drugs; additionally, they can prolong the *in vivo* circulation time of nanoparticles and reduce the probability of macrophage capture. On the basis of the surface modification of nanoparticles, antibodies, polypeptides, and other molecules that are linked to the surfaces of carriers can also be combined with a variety of biological ligands and passively or actively target contrast agents to target sites.[7–9] Small molecular peptides have relatively low molecular weight and low immunogenicity, making them unlikely to induce an immune response. Additionally, their preparation is

relatively simple, making them suitable as targeted molecularly modified nanocarriers. Huh et al.[10] and Hu et al.[11] reported the diagnosis of malignant tumors in a tumor-bearing mouse model using antibody-targeted magnetic nanoparticles. Pan et al.[12] used foliate-mediated ultrasmall superparamagnetic iron oxide for MRI of the human breast cancer cell line MCF-7. The T_2 signal decreased significantly, suggesting that foliate-mediated ultrasmall superparamagnetic iron oxide has a good targeting effect on MCF-7 cells.

Luteinizing hormone-releasing hormone (LHRH), also known as gonadotropin-releasing hormone, is a decapeptide hormone synthesized and secreted by hypothalamic nerve cells. LHRH mainly regulates the generation and release of luteinizing hormone and follicle-stimulating hormone. Current research shows that LHRH is highly expressed on the surfaces of liver, colon, cervical, and ovarian tumors but that its expression is extremely low or absent in the corresponding normal tissues.[13] Therefore, the LHRH receptor can be used as a tumor-specific target for targeted imaging and treatment. In the present study, we prepared Fe_3O_4 nanoparticles using a chemical co-precipitation method coupled with chitosan to prepare LHRH-Fe_3O_4 nanoparticles. The study was performed to explore the application value of LHRH-Fe_3O_4 nanoparticles in targeted imaging and treatment for breast tumors through *in vitro* and *in vivo* experiments.

Materials and methods

Preparation of LHRH-Fe_3O_4 nanoparticles

Fe_3O_4 nanoparticles were prepared by a chemical co-precipitation method, and LHRH-Fe_3O_4 nanoparticles were prepared by adding LHRH coupled with chitosan. A 5-mL LHRH-Fe_3O_4 suspension was centrifuged at low speed for 20 minutes at 12,000 rpm, and the concentration of iron in the supernatant and precipitate was measured by the O-phenanthroline method. The number of LHRH molecules coupled to the surface of chitosan-nanoiron was measured using a bicinchoninic acid protein assay kit. The above LHRH-Fe_3O_4 suspension was placed in a dialysis bag and dialyzed with 5% dextrose solution for 48 hours. The dialysate was changed every 8 hours. The free LHRH and chitosan were dialyzed, and the dialyzed LHRH-Fe_3O_4 solution was diluted with distilled water (1:4). One drop was placed on a coated copper mesh, the excess liquid was removed with filter paper, and the mesh was dipped in 2% tungstic acid for 30 s and then dried. The particle size of LHRH-Fe_3O_4 was then measured by transmission electron microscopy (TEM). After 3 to 5 mL of the solution to be measured was ultrasonically and evenly dispersed and stabilized for 5 minutes, the particle size and size distribution of the prepared LHRH-Fe_3O_4 solution were measured using a laser particle analyzer (Brookhaven Instruments Corporation, Holtsville, NY, USA). The materials required for preparation of LHRH-Fe_3O_4 nanoparticles were supplied by Shanghai Jianglai Biotechnology Co., Ltd. (Shanghai, China).

Uptake of LHRH-Fe_3O_4 nanoparticles by tumor cells

Human MCF-7 breast cancer cells in the logarithmic growth phase were digested by trypsin and inoculated into a six-well plate. When the cells reached 70% to 90% confluence, the supernatant was discarded and LHRH-Fe_3O_4 or Fe_3O_4 was added and cultured for 1, 2, 4, and 8 hours. After harvesting and centrifugation, the cells were dissolved in 100 μL of 65% nitric acid at 95°C for 10 minutes. After the addition of 450 μL of water, the iron concentration of

the samples was determined by atomic emission spectrometry as previously described.[14] The measured iron concentrations were normalized to the cell number of the samples.

Detection of cytotoxicity by cell counting kit[15]

The cells in the logarithmic growth phase were adjusted to a concentration of 1×10^5/mL and then inoculated into a 96-well plate. After the cells were attached, LHRH-Fe_3O_4 or Fe_3O_4 was added to the cells. Three replicates were set in each group. The optical density (OD) at 570 nm in each well was measured by the microplate reader. The growth inhibition rate of the cells was calculated with the following formula: growth inhibition rate (%) = mean OD_{570} in experimental group/mean OD_{570} in control group $\times$ 100%.

Tissue distribution and MRI targeted imaging of LHRH-Fe_3O_4 in tumor-bearing mice

Approximately 1×10^7 MCF-7 cells were collected in 150 μL of serum-free medium and subcutaneously injected into the right axilla of 6-week-old female BALB/c mice (n = 5). LHRH-Fe_3O_4 or Fe_3O_4 was then intravenously administered every 5 days to detect the toxicity *in vivo*. After 30 days of continuous observation, the growth rates were recorded and the hearts, livers, lungs, kidneys, and tumor tissues were removed, fixed in formalin, and stained with hematoxylin–eosin. Pathological changes were observed in various tissues under a microscope. Targeted MRI was performed on day 30 with obvious tumor formation *in vivo*. LHRH-Fe_3O_4 was intravenously injected into the mouse tail vein; head coils were used for scanning to evaluate the contrast effect between the tumor tissues enhanced by LHRH-Fe_3O_4 and normal tissues at 5 minutes and 1, 2, 4, 8, and 24 hours; and the enhancement rates in important organs were calculated.

Statistical analysis

IBM SPSS Statistics for Windows, Version 19.0 (IBM Corp., Armonk, NY, USA) was used to analyze the data. The measurement data are expressed as mean ± standard deviation. The independent-samples *t* test was used in the single-factor variance analysis between groups with $p < 0.05$ indicating statistical significance.

Ethics

All animal experiments and procedures were approved by the Institutional Animal Care and Use Committees of Bengbu Medical College (Permit Number: BMC -20160047).

Results

LHRH-Fe_3O_4 nanoparticle analysis

The particle size of Fe_3O_4 and LHRH-Fe_3O_4 was 8 and 10 nm, respectively, as measured by TEM (Figure 1(a)). The effective particle size of the LHRH-Fe_3O_4 nanoparticles was 10 nm as measured by a particle size analyzer, and the dispersity index was 0.277, indicating that the nanoparticles were uniformly dispersed as shown in Figure 1(b). These findings were basically consistent with the results of TEM.

Uptake of LHRH-Fe_3O_4 nanoparticles by tumor cells

LHRH-Fe_3O_4 or Fe_3O_4 at a final concentration of 1, 10, or 50 μg/mL was co-incubated with MCF-7 cells for 1, 2, 4, or 8 hours. Different levels of iron particles were present in the cells, and the levels of iron particles increased with the concentration and duration of LHRH-Fe_3O_4 or

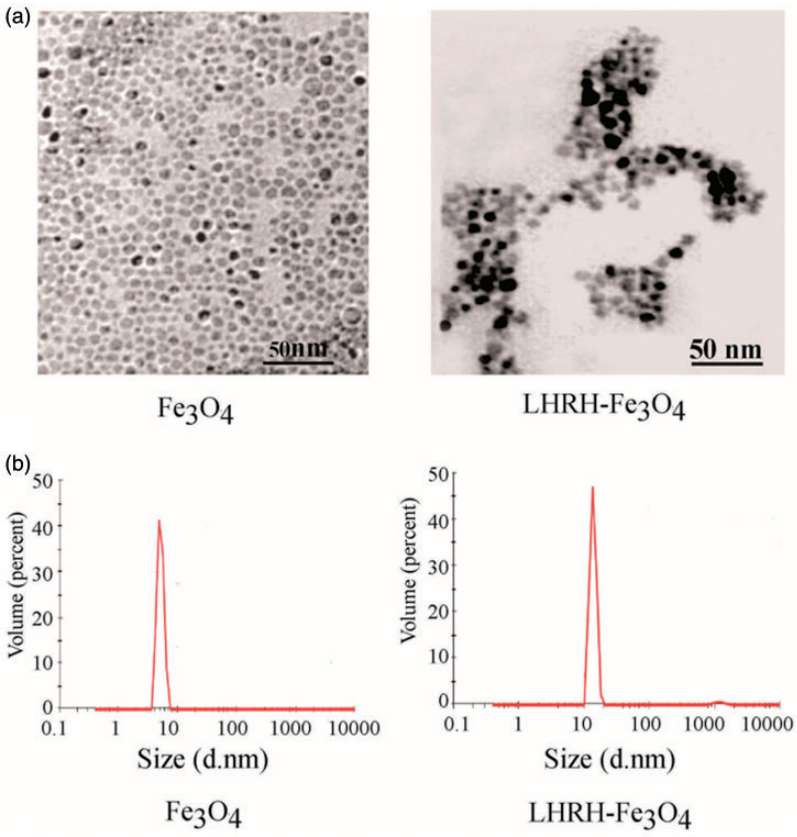

Figure 1. LHRH-Fe_3O_4 nanoparticle analysis. (a) The nanoparticle was analyzed by transmission electron microscopy. (b) The nanoparticle was analyzed by a particle size analyzer. LHRH-Fe_3O_4, luteinizing hormone-releasing hormone–ferrosoferric oxide.

Fe_3O_4 administration. There was significant difference in the levels of iron particles between LHRH-Fe_3O_4 and Fe_3O_4 at 10 and 50 μg/mL ($p < 0.05$) (Figure 2).

Cytotoxicity of LHRH-Fe_3O_4 nanoparticles

In this experiment, the cytotoxicity of the nanomaterial was detected using a Cell Counting Kit-8. As shown in Figure 3, both groups exhibited significant concentration- and time-dependence ($p < 0.05$). There were no significant differences in the inhibition rates at all concentrations between the LHRH-Fe_3O_4 group and Fe_3O_4 group. When the concentration of LHRH-Fe_3O_4 was 50 μg/mL for 72 hours, the inhibition rate in the LHRH-Fe_3O_4 group was 32.26%, while the inhibition rate in the Fe_3O_4 group was 23.12%. This result showed that the nanomaterial had limited cytotoxicity.

Tissue distribution and targeted MRI of LHRH-Fe_3O_4 nanoparticles

Targeted MRI showed that tumor enhancement was not obvious at 5 minutes. After 1 hour, the edge of the tumor began to enhance, the degree of enhancement was

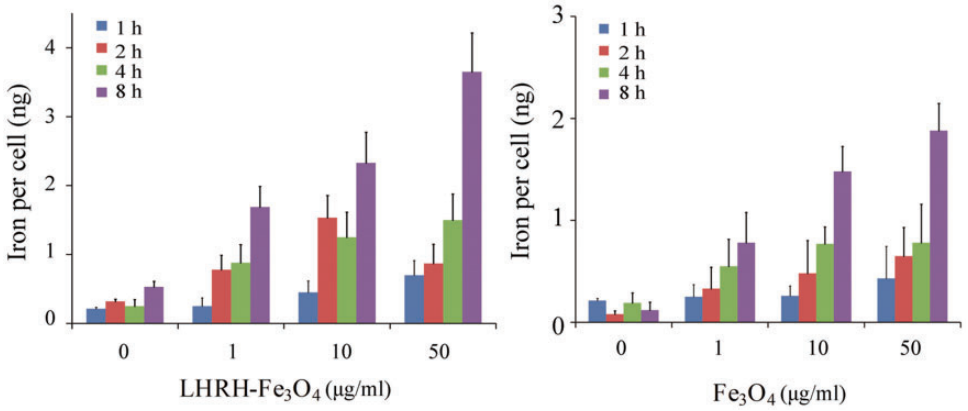

Figure 2. Uptake of LHRH-Fe_3O_4 nanoparticles by tumor cells (n = 5). LHRH-Fe_3O_4, luteinizing hormone-releasing hormone–ferrosoferric oxide.

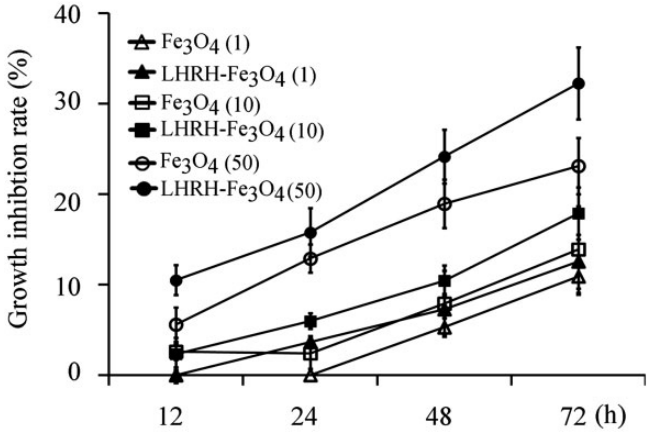

Figure 3. Cytotoxicity of LHRH-Fe_3O_4 nanoparticles was assayed by a Cell Counting Kit-8 (n = 5). LHRH-Fe_3O_4, luteinizing hormone-releasing hormone–ferrosoferric oxide.

low, and the intensification gradually became more obvious as time passed. The intensification diffused from the periphery to the center and peaked at 4 hours. Slight enhancement was observed at 24 hours. The liver tissue of nude mice began to enhance at 5 minutes, peaked at 2 hours, and was not obvious at 4 hours. There was no obvious enhancement in the lung and heart tissues (Figure 4(a)). Moreover, the signal values of each scanning point after injection of LHRH-Fe_3O_4 were paired with the signal value before the LHRH-Fe_3O_4 administration. The results showed that the signal value of the tumor tissue was not significantly different from the injection of LHRH-Fe_3O_4 at 5 minutes but was significantly different at 1, 2, 4, 8, and 24 hours (all $p < 0.05$). The peak tumor enhancement occurred at 4 hours after injection of LHRH-Fe_3O_4, and the maximal enhancement rate was 66.88%.

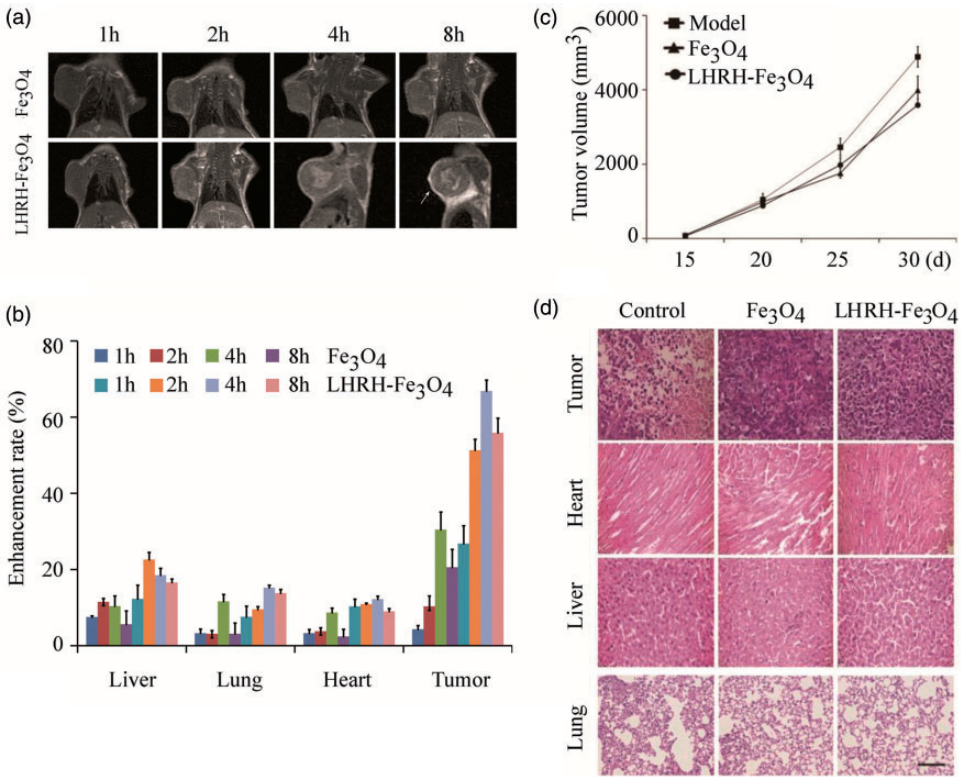

Figure 4. Tissue distribution and targeted magnetic resonance imaging of LHRH-Fe_3O_4 nanoparticles. (a) Magnetic resonance imaging after LHRH-Fe_3O_4 administration. (b) Enhancement rate analysis after LHRH-Fe_3O_4 administration ($n = 5$). (c) Tumor growth to detect the toxicity *in vivo* ($n = 5$). (d) Hematoxylin–eosin staining (200×). LHRH-Fe_3O_4, luteinizing hormone-releasing hormone–ferrosoferric oxide.

These results showed that LHRH-Fe_3O_4 could effectively concentrate on the tumor under the action of a magnetic field (Figure 4(b)).

In our evaluation of toxicity *in vivo*, we found that the growth rates were not significantly different among the model group, LHRH-Fe_3O_4 group, and Fe_3O_4 group after 30 days of continuous observation (Figure 4(c)). After the mice were killed, the tumor, heart, liver, and lung tissues were stained with hematoxylin–eosin. This staining showed no obvious differences in tumor growth and no obvious morphological changes in the heart, liver, and lung tissues (Figure 4(c)), thereby suggesting that LHRH-Fe_3O_4 had no *in vivo* toxicity.

Discussion

LHRH receptors are not expressed in normal cervical connective tissues, squamous epithelium, or hepatocytes, but many LHRH receptors with high affinity are present in cervical adenocarcinoma, colon cancer, and liver cancer, and other types of cancer.[13] At present, some laboratories use LHRH targeting for treatment of tumors. Keller et al.[16] reported that

AN-207, the analog of LHRH (targeted LHRH receptor), was used to treat renal cell carcinoma. Nechushtan et al.[17] made LHRH and PE66 (the mutant of diphtheria toxin) into a fusion protein that exhibited different levels of inhibition on cell growth of colon cancer, ovarian cancer, breast cancer, and liver cancer. Kakar et al.[18] showed that the conjugate of triptorelin and Fe_3O_4 had a significant killing effect on MCF-7 and MDA-MB321 cells, suggesting that LHRH plays a very important role in targeted therapy of tumors. Leuschner et al.[19] found that LHRH-superparamagnetic iron oxide nanoparticles (SPIONs) were highly aggregated in human breast cancer. The monosome SPIONs as controls were mainly aggregated in the liver and had very low affinity for tumors, also suggesting that LHRH-SPIONs can enhance the sensitivity of MRI examination of breast tumors.

In the present study, the particle size of LHRH-Fe_3O_4 nanoparticles was 10 nm, and they could be taken in by human breast cancer cells. The nanomaterial showed limited inhibition of tumor growth *in vitro* and *in vivo*. *In vivo* MRI experiments showed that LHRH-Fe_3O_4 can effectively concentrate on the tumor under the action of a magnetic field, has a good negative enhancement effect that significantly reduces the signal intensity of the T_2 field, and can be used as a contrast agent of the T_2 field.

In conclusion, LHRH-Fe_3O_4 nanoparticles serve the purpose of targeting contrast agents to target sites and are thus expected to be used for targeted imaging and treatment of cancers with high expression of LHRH.

Declaration of conflicting interest

The authors declare that there is no conflict of interest.

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