Evaluation of nano-magnetic fluid on malignant glioma cells

HONGSHENG XU*, HAILIANG ZONG*, CHONG MA, XING MING, MING SHANG, KAI LI, XIAOGUANG HE and LEI CAO

Department of Neurosurgery, Central Hospital of Xuzhou, Affiliated Hospital of Southeast University, Xuzhou, Jiangsu 221009, P.R. China

Received June 16, 2016; Accepted December 13, 2016

DOI: 10.3892/ol.2016.5513

Abstract. The temperature variation rule of nano-magnetic fluid in the specific magnetic field and the effect on the treatment of malignant glioma were examined. The temperature variation of nano-magnetic fluid in the specific magnetic field was investigated by heating *in vitro*, and cell morphology was observed through optical microscopy and electron microscopy. MTT detection also was used to detect the effect of Fe_3O_4 nanometer magnetic fluid hyperthermia (MFH) on the proliferation of human U251 glioma cell line. The Fe_3O_4 nano MFH experiment was used to detect the inhibition rate of the tumor volume in nude mice with tumors. The results of the experiment showed that the heating ability of magnetic fluid was positively correlated with its concentration at the same intensity of the magnetic field. The results also indicated the prominent inhibitory effect of nanometer MFH on the proliferation of glioma cells, which was a dose-dependent relationship with nanometer magnetic fluid concentration. The hyperthermia experiment of nude mice with tumors displayed a significant inhibiting effect of Fe_3O_4 nanometer magnetic fluid in glioma volume. These results explain that iron (II,III) oxide (Fe_3O_4) nanometer MFH can inhibit the proliferation of U251 glioma cells, and has an obvious inhibitory effect on glioma volume, which plays a certain role in the treatment of brain glioma.

Introduction

Malignant glioma is the most common malignant tumor in the brain. It has poor prognosis and is highly heat-resistant to conventional therapy, such as surgery, radiotherapy and chemotherapy (1,2). Therefore, the development of new treatments to improve the prognosis of patients with glioma is the focus and a challenge of the current research. Hyperthermia is a physical therapy, which has fewer side effects compared to chemotherapy. It can be reused many times without taking into account the accumulation of toxicity in the body (3). At present, local hyperthermia is mainly implemented through ultrasound, microwave and radiofrequency, which however, all have some shortcomings (4). Ultrasound cannot pass through the body cavity that holes gas (5). There are also issues such as the reflection of bone, bone resorption and others. Therefore, the treatment is challenging for intracranial tumors; microwave heating cannot be used for deep tumors, and often with side effects like scalding; radiofrequency heating has been widely used in local thermotherapy (6). Radiofrequency does not have a precise positioning, it heats the normal tissue and tumor tissue together and its capacity is affected by various factors (7). In addition, it causes complications like severe fat superheat and skin pain (8). Thermal ablation therapy is able to kill numerous necrosis of tumor tissue instantly due to high temperature. However, various substances released from these necrotic tumor tissues flowing into the blood will cause shock syndrome and lead to high risk complications (9).

Magnetic fluid hyperthermia (MFH) is a new hyperthermia method. Magnetic fluid is injected into the tumor tissues, and the nanometer magnetic fluid will deposit between cells or be engulfed by tumor cells (10). Heating magnetic medium under alternating magnetic field will contribute to shaping local high-heat region and avoid heating normal tissue, which can preserve normal tissues effectively and achieve the best effect (11). The present study explores the effect of Fe_3O_4 magnetic fluid in different concentration under alternating magnetic field on malignant glioma cells *in vitro* and *in vivo*, which provides reference for MFH.

Materials and methods

Main materials

Main reagents. Magnetic fluid was purchased from the magnetic fluid research institute of Beijing Jiaotong University, China. After intermittent sterilization, it was stored at 4˚C. Before using, RPMI-1640 medium (Gibco, Grand Island, NY, USA) and 0.9% saline were used to dilute it to the required concentration.

Cell lines. U251 human glioma cell lines were purchased from the Department of Neurobiology in China Medical University.

Correspondence to: Dr Hongsheng Xu, Department of Neurosurgery, Central Hospital of Xuzhou, Affiliated Hospital of Southeast University, Xuzhou, Jiangsu 221009, P.R. China
E-mail: hongsheng_xu1@163.com

*Contributed equally

Key words: Fe_3O_4 nanometer magnetic fluid, malignant glioma, hyperthermia

Culture conditions were: RPMI-1640 medium of 10% fetal bovine serum (Gibco), a 37˚C, saturated humidity incubator with 5% CO_2. When the cells covered the bottom of the culture bottle, culture medium was discarded and cleaned two times with D-Hank's liquid (Gibco), added with 0.25% trypsin digestive juice (Sigma-Aldrich, St. Louis, MO, USA). After 1 min, trypsin was also discarded before adding fresh medium. Then it was gently pipetted to single cell suspension and its passage dispensed.

Experiment method

Heating experiment of Fe_3O_4 nanometer magnetic fluid in vitro. The magnetic fluid used in the experiments was purchased from the magnetic fluid research institute of Beijing Jiaotong University. Scanning electron microscope (Quanta™ 250; FEI, Hillsboro, OR, USA) an image of the results is shown in Fig. 1, from which we can see that magnetic particles dispersed clearly. Particles that have a diameter of 5-10 nm are uniformly dispersed. The magnetic liquid nano iron concentrations in the experiment were 2, 4, 6 and 8 g/l.

Double distilled water was used to dilute Fe_3O_4 nano magnetic fluid into 2, 4, 6 and 8 g/l, then adding 5 ml into 25 mm flat tubes, respectively, placed in a high-frequency magnetic field with a frequency of 200 KHz, power of 4 kW, and output current of 300 A for 1 h. The starting room temperature was 23˚C. The distance between the tube bottom and high frequency magnetic heating coil center was 0.5 cm. Temperature was detected with TM902C digital thermometer every 5 min. The sample's heating curve was drawn with time as the abscissa and temperature as the ordinate.

Effect of magnetic nanoparticles on glioma in vitro. i) U251 human glioma cell morphology observation: Logarithmic phase cells were seeded into 6-well plates and divided into 5 groups. The control group had only DMEM (Gibco) containing 10% fetal calf serum; then the hyperthermia group was also added, respectively, with 5 ml magnetic fluid at concentrations of 2, 4, 6 and 8 g/l, and underwent irradiation in high frequency alternating magnetic field (Carl Zeiss GmbH, Jena, Germany) for 1 h. The change of cell morphology was observed at 48 h after treatment.

ii) MTT detection for U251 cell proliferation: U251 cells in logarithmic growth phase were prepared into cell suspension ($1x10^5$/ml) as hyperthermia group (4 groups) and control group, respectively. Hyperthermia group was added with nano magnetic fluid of 5 ml in a concentration of 2, 4, 6 and 8 g/l and the control group was added with RPMI-1640 medium for 5 ml and placed into a magnetic field for 1 h. The cell suspensions were inoculated into a 96-well plate, 100 ml per well, each group of 6 wells, for 72 h. The 96-well plate was then removed and the original solution was discarded. A total of 100 ml fresh medium was then added, next the 20 μl MTT (BioSharp, Hefei, China) (5 mg/ml), continued for 4 h. Then the process was stopped, the culture medium containing MTT was discarded, and 100 μl DMSO (Sigma-Aldrich) was added to stop the reaction, then was pipetted gently to completely dissolve the crystallization and to minimize bubbles. Enzyme-linked immunosorbent assay was used to detect absorbance (OD) at a wavelength of 570 nm. The average rate of cell proliferation was calculated as: (1- OD of hyperthermia group/OD of control group) x 100%.

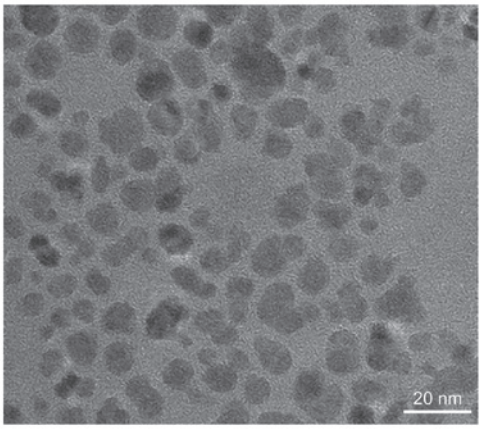

Figure 1. Magnetic nanoparticles in transmission electron microscopy.

Anti-glioma effect of nano magnetic particles in animal experiment. After 1.25 g/l trypsin digestion of U251 human glioma cells in logarithmic growth period, they were suspended in the phosphate buffer saline (PBS) (BioSharp). After counting, concentrations were adjusted. A total of 100 ml of PBS solution containing ~$1x10^7$ cells were injected subcutaneously into 24 male nude mice in the right lower limb. Twenty days later, when the short diameter of subcutaneous tumors were >0.5 cm, mice were randomly divided into the hyperthermia group, magnetic fluid control group and blank control group with 8 rats in each group. In magnetic fluid control group, rats underwent intratumoral injection of 0.3 ml magnetic fluid with a concentration of 4 g/l, and in the MFH group, rats underwent injection of 0.3 ml nano magnetic fluid with concentrations of 2, 4, 6 and 8 g/l, while hyperthermia group underwent anesthesia with intraperitoneal injection of 0.5% sterile sodium phenobarbital solution (BioSharp) in 60 mg/kg. After anesthesia, the tumors were placed on the high frequency magnetic heater for an irradiation of 1 h with an output current of I=300 A, twice with 24 h interval between each time irradiation, and tumor volumes were recorded in 12 h after hyperthermia. Two weeks later, all the animals were sacrificed to remove the tumors and measure their lengths, diameters (a) and short diameters (b), according to the formula of tumor volume inhibition rate = (1- tumor volume in the experimental group/tumor volume in control group) x 100% and tumor volume inhibition rates were calculated. The present study was approved by the Ethics Committee of Xuzhou Central Hospital.

Statistical analysis. Experimental results were analyzed with SPSS 18.0 (IBM Corp., Armonk, NY, USA) in a t-test or in a variance analysis, and $P<0.05$ was considered to indicate a statistically significant difference.

Results

Heating experiment of Fe_3O_4 nanometer magnetic fluid in vitro. Experimental results showed that the heating capacity of magnetic fluid was positively correlated with the concentration of magnetic fluid. Thus, the greater the concentration of magnetic fluid was at a certain magnetic field intensity, the stronger was the heating capacity. But

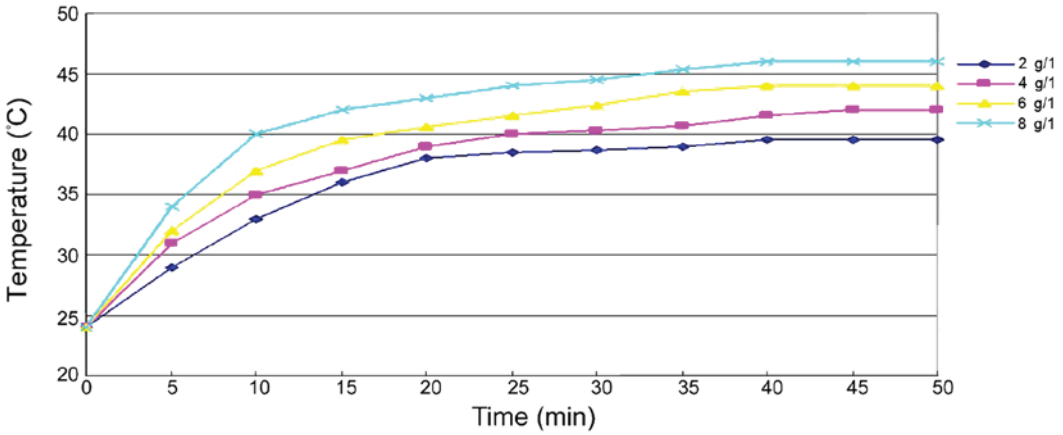

Figure 2. Changes of temperatures with different concentrations in specific alternating magnetic field.

Table I. MTT results of different concentrations of Fe_3O_4 nanometer magnetic fluid hyperthermia in U251 cells (mean ± standard deviation).

Groups	Control group	2 g/l	4 g/l	6 g/l	8 g/l
OD value	0.95±0.01	0.75±0.02	0.51±0.03	0.37±0.01	0.12±0.02
Inhibition rate (%)		21.5	46.3	61.1	87.4

P<0.05 in treatment groups compared with the control group.

Table II. Inhibiting effect of Fe_3O_4 nanometer magnetic fluid hyperthermia in different concentrations on tumor volume (mean ± standard deviation).

Groups	Blank control group	Magnetic fluid control group	2 g/l	4 g/l	6 g/l	8 g/l
Tumor volume (cm^3)	19.58±3.53	17.24±2.82	12.17±3.21	9.88±2.14	5.69±2.03	2.43±1.17
Inhibition rate (%)		11.9	37.7	49.5	69.6	12.4

P<0.05 in hyperthermia group with single factor analysis of variance, compared with control group.

there was a common rule: Temperature grew rapidly in the first 20 min, but stayed at a constant level 40 min later (Fig. 2).

Effect of magnetic nanoparticles on glioma in vitro. i) U251 human glioma cell morphology observation: After nano MFH, human glioma cells were observed through inverted microscopy. Cells in the control group were the same size without cell rupture or cell debris, and with a greater number of cells which appeared well attached. In the hyperthermia group, the temperature increased in response to an increase in Fe_3O_4 nanometer magnetic fluid concentration. Normal cells gradually decreased while necrotic cells and cell debris increased gradually with less attachment to the walls, with more magnetic material deposition. Electron microscopy showed that in the hyperthermia group, cell chromatin was condensed with cytoplasmic vacuoles, and apoptotic bodies were formed; nanometer materials deposited in the nucleus, cytoplasm and lysosomes which were confirmed as nanoparticles analyzed through energy spectrum analysis.

ii) MTT detection of U251 cell proliferation: Nano MFH had a significant inhibiting effect on the proliferation of glioma cells with a dose-dependent relationship with nano magnetic fluid concentration. The difference between the groups was statistically significant (P<0.05) (Table I).

Anti-glioma effect of nano magnetic particles in animal experiment. The tumors in the control group increased with the prolongation of treatment time, with smooth surfaces and no hemorrhage or necrosis. Tumors in the hyperthermia group decreased gradually during the whole cycle of treatment, with hemorrhage and necrosis by histological examination. With the rise of the concentration, the inhibitory effect of magnetic fluid gradually increased. The difference of tumor volumes between three groups was statistically significant (P<0.05) (Table II). It was shown that MFH has a certain therapeutic effect on

glioma cells, and curative effect was enhanced with higher magnetic fluid concentration.

Discussion

Glioma, which is originated from the neuroectoderm in the central nervous system, is the most common primary tumor in human intracranial tumors and a cause of death (2). Because of the infiltrating invasive growth of glioma and the vague boundary between tumor tissue and normal brain tissue, even surgical treatment results in 5-year survival rate of <5% and with a high risk of recurrence (1). These led us to identify a new treatment option for glioma.

Jordan *et al* performed an antitumor study on glioma model of rats with MFH method (12,13). The Fe_3O_4 magnetic fluid covered by amino silane was used and injected into the tumor tissue within the magnetic field (100 kHz, 18 kA, m-1) for an irradiation of 30 min, and then the tumor tissue temperature reached 43-47˚C. Compared with the control group, the survival rate of the model was significantly prolonged. Pathological examination showed a large necrotic area of tumor tissue, and nano magnetic particles were uniformly dispersed in that tissue. Maier-Hauff *et al* (14) injected magnetic particles into the glioma tumor of patients, and the temperature of the interior of the tumor reached 44.6 Yi (42.4-49.5 Yi) in an alternating magnetic field. Patients could tolerate the treatment without obvious side effects. MFH can be used in applications for deep intracranial tumors such as glioma, which indicated its safety. The present study prepared nano magnetic material with high heating-yield, in order to reduce the amount of the material. The heating experiment *in vitro* showed that in a certain magnetic field intensity, the magnetic fluid heating capacity was positively correlated with magnetic fluid concentration in the magnetic field, and cell morphology was observed by optical microscopy and electron microscopy. MTT detection of Fe_3O_4 nanometer MFH was used to detect the proliferation rate of U251 glioma cell line. The result indicated a significant inhibitory effect on the proliferation of glioma cells in a dose dependent manner compared to nano magnetic fluid concentration. Liver cancer inhibition rate of Fe_3O_4 nanometer MFH was detected by rat hyperthermia experiment.

Hyperthermia has not been really practical in clinical application, mainly due to the scarcity of detailed study of magnetic nanoparticles in human metabolism and clearance mechanism and toxicity. Hyperthermia could not completely inhibit tumor and it greatly restricts the application of MFH in the treatment of tumors (15,16). We also need many further experiments to demonstrate the safety of application of magnetic fluid in other aspects of the animal experiment and treatment, which may improve the efficacy and reduce adverse reactions that can be used in the clinical diagnosis and treatment in the future.

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