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Nanosized As_2O_3/Fe_2O_3 complexes combined with magnetic fluid hyperthermia selectively target liver cancer cells

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Abstract

AIM: To study the methods of preparing the magnetic nano-microspheres of Fe_2O_3 and As_2O_3/Fe_2O_3 complexes and their therapeutic effects with magnetic fluid hyperthermia (MFH).

METHODS: Nanospheres were prepared by chemical co-precipitation and their shape and diameter were observed. Hemolysis, micronucleus, cell viability, and LD_{50} along with other *in vivo* tests were performed to evaluate the Fe_2O_3 microsphere biocompatibility. The inhibition ratio of tumors after Fe_2O_3 and As_2O_3/Fe_2O_3 injections combined with induced hyperthermia in xenograft human hepatocarcinoma was calculated.

RESULTS: Fe_2O_3 and As_2O_3/Fe_2O_3 particles were round with an average diameter of 20 nm and 100 nm as observed under transmission electron microscope. Upon exposure to an alternating magnetic field (AMF), the temperature of the suspension of magnetic particles increased to 41-51℃, depending on different particle concentrations, and remained stable thereafter. Nanosized Fe_2O_3 microspheres are a new kind of biomaterial without cytotoxic effects. The LD_{50} of both Fe_2O_3 and As_2O_3/Fe_2O_3 in mice was higher than 5 g/kg. One to four weeks after Fe_2O_3 and As_2O_3/Fe_2O_3 complex injections into healthy pig livers, no significant differences were found in serum AST, ALT, BUN and Cr levels among the pigs of all groups ($P > 0.05$), and no obvious pathological alterations were observed. After exposure to alternating magnetic fields, the inhibition ratio of the tumors was significantly different from controls in the Fe_2O_3 and As_2O_3/Fe_2O_3 groups (68.74% and 82.79%, respectively; $P < 0.01$). Tumors of mice in treatment groups showed obvious necrosis, while normal tissues adjoining the tumor and internal organs did not.

CONCLUSION: Fe_2O_3 and As_2O_3/Fe_2O_3 complexes exerted radiofrequency-induced hyperthermia and drug toxicity on tumors without any liver or kidney damage. Therefore, nanospheres are ideal carriers for tumor-targeted therapy.

Key words: Liver cancer; Magnetic fluid hyperthermia; Nanoparticle; As_2O_3

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors in China, and the incidence has increased in recent years. Current therapeutic options remain unsatisfactory for most patients. Surgical resection has been recognized as the most effective method for the treatment of hepatocarcinoma, but it is only indicated for a small number of hepatocarcinoma patients[1,2]. Therefore, it is crucial to identify a new method to treat hepatocarcinoma.

In recent years, radiofrequency-induced hyperthermia has increasingly attracted attention for the generation of heat in a desired zone, even in tumors deeply located inside a patient's body. During exposure to alternating

magnetic field (AMF), magnetic particles can absorb energy and transform it into heat at temperatures of 42-45℃, at which tumor cells are very sensitive[3]. In addition, the use of magnetic nanospheres, which can carry the magnetic particles into the tumor cells very easily, can greatly enhance the effects of the thermotherapy. In our research, we attempted to prepare a kind of new magnetic material that contained As_2O_3. We transformed the energy of the radio waves into heat to kill tumor cells and explored the therapeutic effects of nanospheres for the treatment of hepatocarcinoma.

MATERIALS AND METHODS

Materials

As_2O_3 and dimethyl sulfoxide (DMSO) was purchased from Sigma. RPMI-1640 medium was obtained from GIBCOL-BRL. Newborn calf serum was from Si-Ji-Qing Biotechnology Co. (China). HEPES, Trypsin and methyl thiazolyl tetrazolium (MTT) were purchased from AMRESCO. The transmission electron microscope (TEM) used was a H-600 model (Hitachi, Japan) and the scanning electron microscope (SEM) model was JEOL JSM-6360LV (Japan). The energy dispersive spectrometer (EDS) was purchased from Thermo NORAN Vantage (USA).

L929 cells (human fibroblast cell line) and SMMC-7721 cells (human liver cancer cell line) were purchased from the Institute of Biochemistry and Cell Biology, Shanghai Institute of Biological Sciences, Chinese Academy of Sciences.

BALB/C nude mice (male, 10-wk-old) were purchased from the Lakes Animal Experimental Center of the Institute of Biochemistry and Cell Biology, Shanghai Institute of Biological Sciences, Chinese Academy of Sciences.

Methods

Preparation and characteristics of Fe_2O_3 and As_2O_3/Fe_2O_3 nanoparticles: Fe_2O_3 magnetic nanoparticles were prepared according to the method previously described[4]. Fe_2O_3 magnetic nanoparticles were added into a solution of As_2O_3 (0.01 mg/mL, pH = 5, adjusted by acetic acid) under a condition of supersonic dispersion. After 30 min at 80℃, the products were centrifuged at 2000 r/min for 10 min, rinsed twice by absolute alcohol, and then dried in a vacuum. The diameter and composition of Fe_2O_3 and As_2O_3/Fe_2O_3 were examined under TEM and EDS.

A heating test was performed to detect the thermodynamic characteristics of the magnetic particles. Various doses of Fe_2O_3 and As_2O_3/Fe_2O_3 particles were decentralized in 0.9% NaCl. The concentrations of Fe_2O_3 were 2, 4, 6 and 8 g/L. Two milliliters of nanoparticle fluid was then added to a flat-bottomed cuvette to reach a level of 5 mm from the bottom of the cuvette and in the center of the hyperthermia-coil of a high frequency electromagnetic field. The output electric current was 300 A, and the fluid was heated for 1 h with temperature measurement at 5 min intervals.

Biocompatibility study of Fe_2O_3 nanoparticles: MTT assay, hemolytic test, and micronucleus assay were performed to test the *in vitro* cytotoxicity of Fe_2O_3 nanoparticles. To perform the MTT assay, L929 cells were cultured in RPMI-1640 media supplemented with 10% heat-inactivated calf serum, penicillin (100 U/mL) and streptomycin (100 mg/mL) and grown in the presence of 5% CO_2 at 37℃. Cells were seeded in a 96-well plate and treated with 200 μL Fe_2O_3 nanoparticle fluid at various concentrations (100%, 75%, 50% and 25%) for 48 h and with 5 μmol/L of As_2O_3 as a positive control. Subsequently, 20 μL (5 g/L) MTT was added to the cells in each well and incubated for 4 h at 37℃. Culture media was discarded and 150 μL of DMSO was added and subjected to vibration for 10 min. The absorbance (A) value was measured at a wavelength of 493 nm. The cell relative growth rate (RGR) was calculated as follows: (A of experimental group/A of control group) × 100%.

For the hemolytic test, 50 mL of Fe_2O_3 and As_2O_3/Fe_2O_3 was centrifuged at 2000 r/min for 10 min 3 times, then suspended and incubated at 37℃, and after 30 min a liquid-extract was obtained. Ten milliliters of 0.9% NaCl and 10 mL of double distilled water were used as negative (0% hemolysis) and positive (100% hemolysis) controls, respectively. Each group contained three tubes. Diluted anticoagulated rabbit blood (0.2 mL) was added to each tube, which had been pre-heated at 37℃ for 30 min. Contents of all the tubes were incubated in a water bath at 37℃ for 60 min. All tubes were centrifuged at 2500 r/min for 5 min and the supernatant was taken to estimate free hemoglobin. Absorbance was measured and recorded at 540 nm. In general, the optical density was 0.8 ± 0.3 in positive control groups and was no more than 0.03 in negative control groups. The hemolysis rate (HR) was calculated as follows: HR (%) = (A of experimental group - A of negative control group)/(A of positive control group - A of negative control group) × 100%.

For the micronucleus assay, 60 mice were randomly divided into six groups, with five females and five males in each group. Animals were injected intraperitoneally with 100 g/L of Fe_2O_3 or As_2O_3/Fe_2O_3 (40 mg/kg) twice at a 24 h interval. The negative group (with 0.9% NaCl) and positive group (with CT × 40 mg/kg) were set as control groups. Six hours after the second injection, all the mice were killed. The thighbone marrows were extracted for smears, methanol-fixed for 5 min, then dyed with Giemsa for 15 min. For each smear, 1000 polychromatic erythrocytes (PEC) were counted, and the number of PEC containing micronucleus was calculated (MN). Poisson distribution verified the statistical difference of each group.

We also studied the *in vivo* histotoxicity of nanoparticles. The Kun Ming mice were divided into 15 groups randomly with five females and five males in each group. Various amounts of 100 g/L Fe_2O_3 and As_2O_3/Fe_2O_3 nanoparticles were intraperitoneally injected into each mouse of seven groups at 1.25, 1.75, 2.5, 3.5, 5,

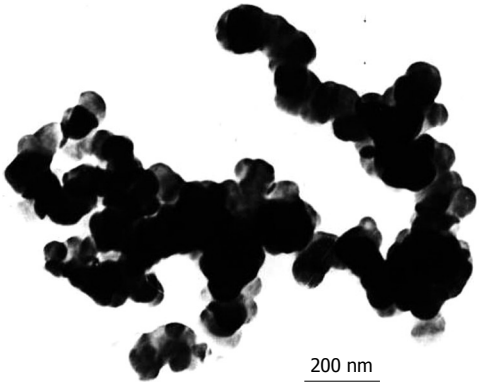

Figure 1 Shape of As_2O_3/Fe_2O_3 observed under TEM.

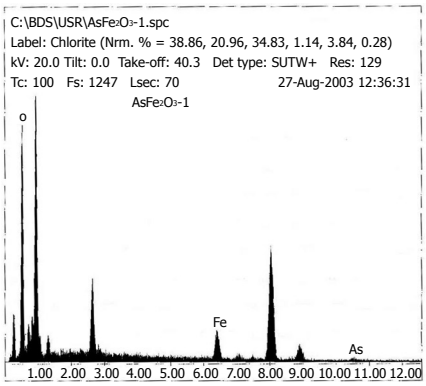

Figure 2 EDS results of As_2O_3/Fe_2O_3.

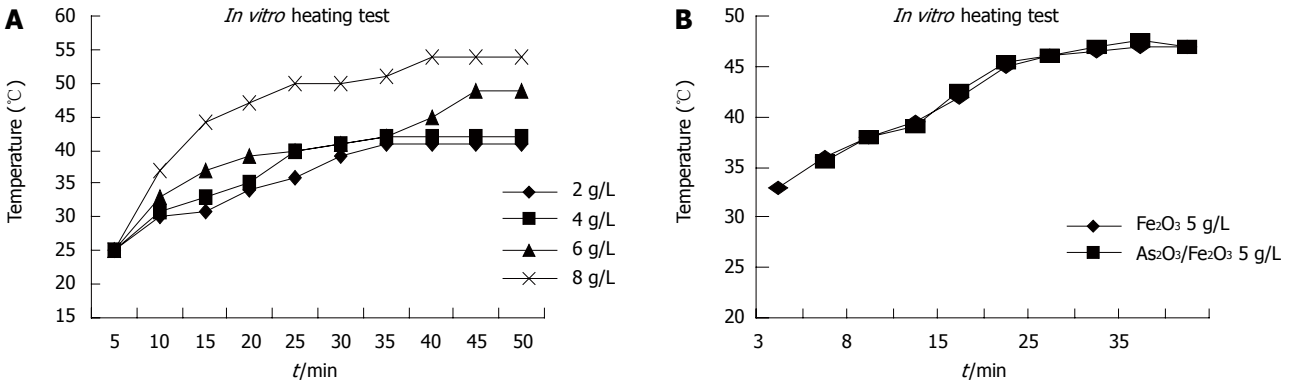

Figure 3 Thermodynamic test. A: Various doses of Fe_2O_3; B: The concentration of Fe_2O_3 is 5 g/L.

7.07 and 10 g/kg according to their weight. The negative control group was injected with the same volume of 0.9% NaCl, and the mice were observed in the following 15 d. The median lethal dose (LD_{50}) was evaluated by the Karber method.

Sixteen healthy pigs were divided randomly into four groups (control group, Fe_2O_3 low dose, Fe_2O_3 high dose, and the As_2O_3/Fe_2O_3 group). Fe_2O_3 or As_2O_3/Fe_2O_3 (10 g/L) was injected into the liver of pigs in the experimental groups. Four pigs from each group were killed from 1 to 4 wk after injection. Serum AST, ALT, BUN and Cr were measured. Livers were harvested and dissected into 1 mm^3 specimens. Subsequently, the samples were fixed in 4% glutaraldehyde and were prepared into ultrathin sections (60 nm) to be examined under TEM and EDS.

Therapeutic effect of As_2O_3/Fe_2O_3 in combination with MFH on xenograft liver cancer

Inhibition of SMMC-7721 cell proliferation was measured by MTT assay according to the method described above. Xenograft tumors were induced in the subcutaneous tissue around the right shoulder of nude mice with SMMC-7721 cells. Once the tumor diameter increased to 0.2-0.4 cm, mice were divided into 6 groups: (1) the control (sterile 0.9% NaCl); (2) As_2O_3 (5 μmol/L As_2O_3); (3) Fe_2O_3 (5 g/L Fe_2O_3); (4) As_2O_3/Fe_2O_3 (5 g/L Fe_2O_3); (5) $Fe_2O_3$with hyperthermia; and (6) As_2O_3/Fe_2O_3 with hyperthermia. Each group contained eight mice. They were injected into the tumors at 1/2 of the volume of the tumor. The tumors of the mice in groups 5 and 6 were exposed to a high-frequency alternating magnetic field and irradiated for 30 min. The treatment was given three times at 24 h intervals. After 45 d, all the mice were killed. The weight and volume inhibitory rates of the tumor were calculated as follows: IW = (1 - the weight of tumor of experimental group/ the weight of tumor of control group) ×100%; Iv = (1 - the volume of tumor of experimental group/the volume of tumor of control group).

Statistical analysis

Values were expressed as mean ± SD. The data were analyzed with SPSS 11.5 and SAS 10.0 software packages. Differences in the results were considered statistically significant when $P < 0.05$.

RESULTS

Characteristics of Fe_2O_3 and As_2O_3/Fe_2O_3 nanoparticles

Under TEM, the nanospheres appeared to be roughly spherical, brown particles that could be suspended stably in water with good dispersibility. The diameter of Fe_2O_3 particles was about 20 nm, and the diameter of As_2O_3/Fe_2O_3 particles was about 100 nm as shown in Figure 1. The EDS result verified that the nanoparticles contained magnetic particles and As_2O_3 (Figure 2).

Figure 3A shows the thermodynamic tests of various

Table 1 Results of MTT test (mean ± SD)

Group	Absorbance value	RGR (%)	Cytotoxicity gradations
Negative control	0.4671 ± 0.0103	100.00	0
25% Fe_2O_3	0.4793 ± 0.0210	102.63	0
50% Fe_2O_3	0.4501 ± 0.0101	96.39	0
100% Fe_2O_3	0.4453 ± 0.0108	95.35	0
25% As_2O_3/Fe_2O_3	0.4373 ± 0.0210	93.64	0
50% As_2O_3/Fe_2O_3	0.1788 ± 0.0247	38.29	3
100% As_2O_3/Fe_2O_3	0.1273 ± 0.0073	27.26	3
As_2O_3 (5 μmol/L)	0.1322 ± 0.0090	30.12	3
Positive control	0.0733 ± 0.0050	15.70	4

Table 3 Results of micronucleus assay (n = 10)

Groups	PEC	PEC containing MN	MN-formation rates (%), mean ± SD
Negative control	10000	24	0.24 ± 1.58
Positive control	10000	241	24.1 ± 4.63
Fe_2O_3	10000	18[a]	0.28 ± 1.40
As_2O_3/Fe_2O_3	10000	29[a]	0.26 ± 1.65

[a]P < 0.05 compared with negative control group.

Table 5 Results of acute toxicity test of As_2O_3/Fe_2O_3

Groups	Dose (g/kg)	Log	N	Deaths (N)	Mortality % (p)	Survival % (q)	p × q
1	10.00	1.000	10	10	100	0	0.00
2	7.07	0.849	10	5	50	50	0.25
3	5.00	0.699	10	3	30	70	0.21
4	3.50	0.544	10	3	30	70	0.21
5	2.50	0.398	10	1	10	90	0.09
6	1.75	0.243	10	0	0	100	0.00
		i = 0.15				∑p = 2.2	

$lgLD_{50}$ = 1-0.15 (2.2-0.5) = 0.745, Sm = 0.0414, As_2O_3/Fe_2O_3 Lg LD_{50} and its 95% CI: 0.745 ± 1.96 × 0.0414 = 0.745 ± 0.0811, As_2O_3/Fe_2O_3 LD_{50} and its 95% CI: 5.56 g/kg (4.56-6.70 g/kg).

doses of magnetic nanoparticles. Fe_2O_3 particles were decentralized in 0.9% NaCl and exposed to a high-frequency alternating electromagnetic field (output current equal to 300 A) for 60 min. The temperature rose rapidly within 5 min and slowly continued to increase from 5-40 min, and remained stable after 40 min. The temperature of the magnetic fluid (MF) rose from 41℃ to 51℃, depending on the different concentrations. The results showed that Fe_2O_3 nanoparticles had good power absorption capabilities in the high-frequency alternating electromagnetic field, and had strong magnetic responsiveness. We selected a suitable temperature range (42-46℃) for tumor hyperthermia[5] by adjusting the concentration of Fe_2O_3. We chose a concentration of 5 g/L for Fe_2O_3, and at this concentration, the temperature rose to 46℃ in MFH (Figure 3B).

Biocompatibility study of Fe_2O_3 and As_2O_3/Fe_2O_3 nanoparticles

MTT assay: The RGR of L929 cells treated with 25%, 50%, and 100% of liquid-extract of Fe_2O_3 were 102.63%, 96.39%, and 95.35%; for As_2O_3/Fe_2O_3 were 93.64%, 38.29%, and 27.26%, respectively. The value of the As_2O_3 group (5 μmol/L) was 30.12% (Table 1). The results corresponded to the cellular morphological changes observed under an inverted microscope.

Table 2 Results of hemolytic test of Fe_2O_3 and As_2O_3/Fe_2O_3 liquid extracts

Group	Absorbance (A)			Average	Hemolysis
	1	2	3	A	rate (HR, %)
Negative control	0.234	0.234	0.236	0.235	
Fe_2O_3 extract	0.232	0.235	0.233	0.233	0.00
As_2O_3/Fe_2O_3 extract	0.246	0.246	0.246	0.246	0.77
Positive control	1.688	1.776	1.521	1.662	

Table 4 Results of acute toxicity test of Fe_2O_3

Groups	Dose (g/kg)	Log	N	Deaths (N)	Mortality % (p)	Survival % (q)	p × q
1	10.00	1.000	10	10	100	0	0.00
2	7.07	0.849	10	4	40	60	0.24
3	5.00	0.699	10	4	40	60	0.24
4	3.50	0.544	10	2	20	80	0.16
5	2.50	0.398	10	0	0	100	0.00
6	1.75	0.243	10	1	10	90	0.09
7	1.25	0.097	10	0	0	100	0.00
		i = 0.15				∑p = 2.1	

$lgLD_{50}$ = 1-0.15 (2.1-0.5) = 0.76, Sm = 0.04, Fe_2O_3 Lg LD_{50} and its 95% CI: 0.76 ± 1.96 × 0.04 = 0.76 ± 0.079, Fe_2O_3 LD_{50} and its 95% CI: 5.75 g/kg (4.80-6.90 g/kg).

Hemolytic test: The Absorbance of each group was observed at 545 nm. As shown in Table 2, the HR of Fe_2O_3 and As_2O_3/Fe_2O_3 nanoparticles was 0% and 0.77%, which is far less than the standard 5% that indicates a hemolytic reaction.

Micronucleus assay: The MN formation rates of Fe_2O_3, As_2O_3/Fe_2O_3, the negative control and the positive control groups were 0.28%, 0.26%, 0.24%, and 24.1%, respectively (Table 3).

Median lethal dose (LD_{50}) determination: The mice receiving various doses were observed over the subsequent 15 d and the experimental animals died in succession. The LD_{50} was evaluated by the Karber method. The LD_{50} of mice receiving Fe_2O_3 and As_2O_3/Fe_2O_3 were 5.75 g/kg and 5.56 g/kg, respectively (Tables 4 and 5).

Biocompatibility study in pigs: One to four weeks after injection of Fe_2O_3 and As_2O_3/Fe_2O_3 into healthy pig livers, no significant differences were found in serum AST, ALT, BUN and Cr levels among pigs of all groups (P > 0.05), and no obvious pathological alterations were observed (Table 6). EDS examination revealed that in the As_2O_3/Fe_2O_3 complex group, numerous black

Table 6 Results of biocompatibility study *in vivo* (mean ± SD)

Groups	TB	ALT	AST	Bun	Cr
Control	4.8 ± 1.67	41.5 ± 10.08	92.25 ± 46.81	3.3 ± 0.36	86.5 ± 6.19
Fe_2O_3 low dose	8.025 ± 2.70	44.25 ± 16.35	87 ± 29.01	3 ± 0.99	83.25 ± 4.65
Fe_2O_3 high dose	6.45 ± 2.97	34.5 ± 9.61	71.25 ± 35.64	2.475 ± 0.46	77.5 ± 15.44
As_2O_3/Fe_2O_3	7.93 ± 2.66	47.25 ± 14.19	52.75 ± 5.5	2.5 ± 0.25	92.25 ± 19.69

Compared with control group: $P > 0.05$.

Table 7 Volume and mass inhibitory rate of xenograft liver cancer in nude mice after treatment

Groups	Tumor mass (g, mean ± SD)	Mass inhibitory rate (%)	Tumor volume (cm^3, mean ± SD)	Volume inhibitory rate (%)
Control	0.6145 ± 0.2296	0.00	0.7195 ± 0.3231	0.00
As_2O_3	0.5885 ± 0.1628	5.86	0.6015 ± 0.2282	16.40
Fe_2O_3	0.5365 ± 0.2792	12.69	0.5997 ± 0.2518	16.65
As_2O_3/Fe_2O_3	0.4548 ± 0.2591	26.04	0.6252 ± 0.4034	21.45
Fe_2O_3 with MFH	0.1921 ± 0.0395	68.74[b]	0.2373 ± 0.0874	67.02[b]
As_2O_3/Fe_2O_3 with MFH	0.1057 ± 0.0510	82.79[b,c]	0.1183 ± 0.0726	83.56[b,c]

[b]$P < 0.01$ *vs* control group; [c]$P < 0.05$ *vs* Fe_2O_3 with MFH group.

nanosized As_2O_3/Fe_2O_3 complexes had accumulated in the liver tissue of pigs.

Therapeutic effect of Fe_2O_3 and As_2O_3/Fe_2O_3

Morphological changes of apoptotic SMMC-7721 cells: The morphological changes of SMMC-7721 cells after treatment were observed under inverted microscopy. As shown in Figure 4, cells in the control and the Fe_2O_3 groups exhibited a normal shape, clear edge, and no cell fragmentation (Figure 4A). In the groups of As_2O_3, the As_2O_3/Fe_2O_3, the Fe_2O_3 and As_2O_3/Fe_2O_3 combined with MFH, the SMMC-7721 cells became small and global. Some shrunk and even a portion of the cells were suspended, which revealed the typical changes associated with apoptosis (Figure 4B-D). The results showed that both As_2O_3 (5 μmol/L) and MFH (at 46℃) could damage liver cells by inducing apoptosis.

Inhibition of SMMC-7721 cell proliferation after treatment with Fe_2O_3 and As_2O_3/Fe_2O_3 combined with MFH: The results of MTT assay are shown in Figure 5. The cell survival rates of cells treated with Fe_2O_3 and As_2O_3/Fe_2O_3 combined with MFH were 19.66% and 19.95%, respectively, which was statistically different from the negative group ($P < 0.01$). So, the therapeutic effect of nanosized As_2O_3/Fe_2O_3 complexes in combination with MFH on SMMC-7712 cells is much better than that of As_2O_3 or Fe_2O_3 nanoparticles alone.

***In vivo* inhibitory effect of Fe_2O_3 and As_2O_3/Fe_2O_3 combined with MFH on xenograft liver cancer in nude mice:** Animal experiments showed that tumors in the experimental groups became smaller (Figure 6). The mass and volume inhibitory ratio of the As_2O_3/Fe_2O_3 combined with MFH were IM = 82.79% and IV = 83.56%, respectively, which was much higher than that of the other groups (Table 7). Compared with control and experimental groups, each group was markedly different from the controls ($P < 0.01$). Histological examination in the As_2O_3/Fe_2O_3 group revealed that there was an accumulation of black nanosized As_2O_3/Fe_2O_3 particles at the stroma in the margin of the tumors. Many of the tumor cells disappeared at the site adjacent to this accumulation, and a necrotic zone was found surrounding the material (Figure 6).

DISCUSSION

As_2O_3, a traditional Chinese medicine, plays an important role in the treatment and research for human cancers such as acute promyelocytic leukemia (APL), myeloid leukemia, gastric cancer, breast cancer, neuroblastoma and esophageal carcinoma, as well as head and neck cancers[6-8], however, there are many limitations to its use due to its form. Patients treated with As_2O_3 suffered from acute and chronic side effects such as gastrointestinal reactions, which are often severe or fatal[9-11]. Moreover, it has been generally considered to be an extremely effective environmental cocarcinogen for some human malignancies, especially for skin and lung cancer[12]. Therefore, enhancing the curative effect and reducing the toxicity of As_2O_3 by changing its form is of great importance.

Hyperthermia for tumor therapy has been a long-standing modality. At present, thermotherapy is commonly used clinically with such applications as radiofrequency, microwave and lasers, all of which have many limitations for tumor hyperthermia. In 1997, Jordan[13] discovered that a nanoscaled magnetic fluid could be absorbed with much higher power in an alternating magnetic field, and used to treat disease or tumors. This treatment is named "Magnetic Fluid Hyperthermia (MFH)". MFH has a high ability to target and localize thermogenic actions. Therefore,

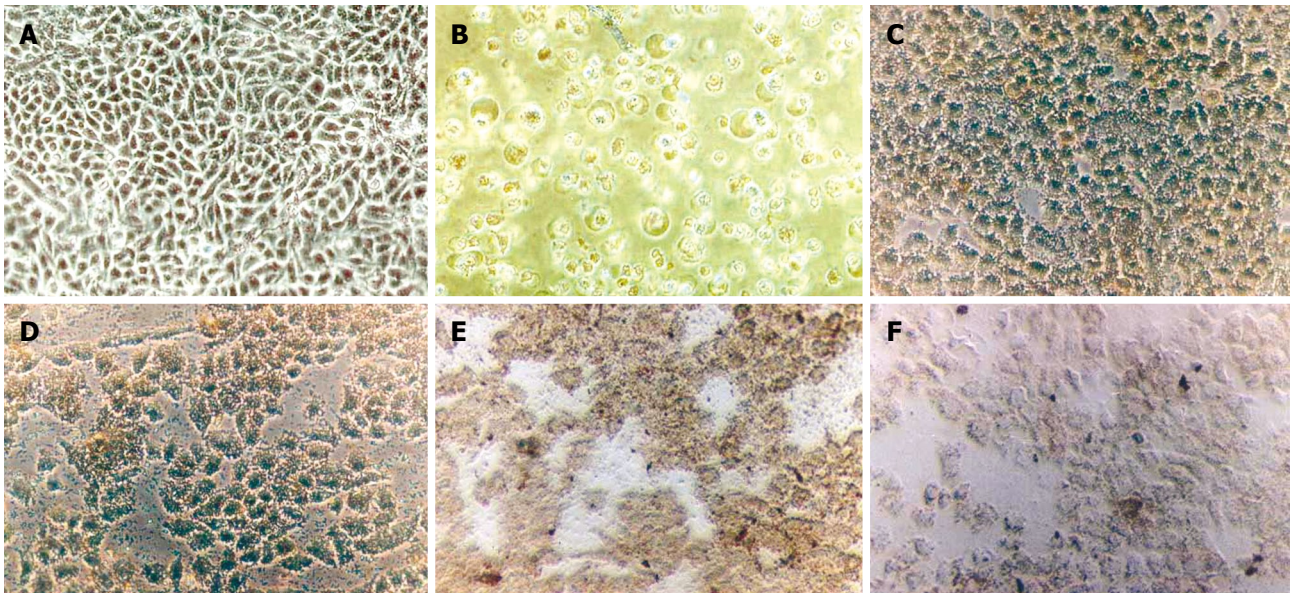

Figure 4 Inverted microscopy of SMMC-7721 cells treated by different methods. A: Negative control group; B: As_2O_3 (5 μmol/L) group; C: Fe_2O_3; D: As_2O_3/Fe_2O_3 group; E: Fe_2O_3 combined with MFH group; F: As_2O_3/Fe_2O_3 combined with MFH group (× 200).

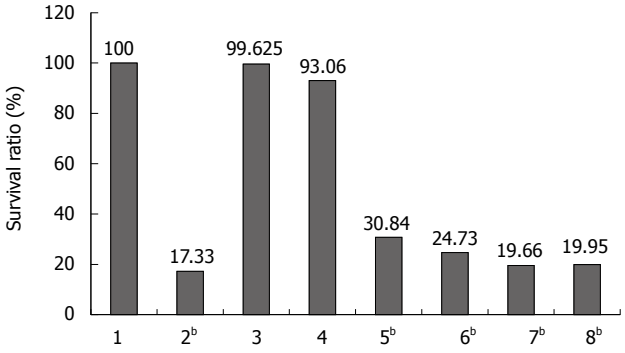

Figure 5 Results of MTT assay of SMMC-7721 cells treated with Fe_2O_3 and As_2O_3/Fe_2O_3 combined with MFH. 1: Negative control; 2: Positive control; 3: MFH alone; 4: Fe_2O_3 (5 g/L); 5: As_2O_3/Fe_2O_3 (5 g/L); 6: As_2O_3 (5 μmol/L); 7: Fe_2O_3 (5 g/L) combined with MFH; 8: As_2O_3/Fe_2O_3 (5 g/L) combined with MFH. ($^bP < 0.01$ *vs* negative group).

Figure 6 Morphological changes of tumors from tumor-bearing nude mice treated by various methods. 1: Control; 2: As_2O_3; 3: Fe_2O_3; 4: As_2O_3/Fe_2O_3; 5: Fe_2O_3 combined with MFH; 6: As_2O_3/Fe_2O_3 combined with MFH.

tissue without magnetic particles would not be damaged.

Nanoparticles combined with MFH may be a potential method to treat tumors. In our study, As_2O_3/Fe_2O_3 complexes were prepared as a new magnetic material. Observed under TEM, they are round or elliptical, disperse well and are about 100 nm in diameter. In addition, they have good power absorption capabilities in a high-frequency alternating electromagnetic field. The temperature can rapidly reach 46℃ within 5 min, which can kill tumor cells while having little effect on normal cells.

However, the biomaterials would be in direct contact with tissues and cells when introduced into the body, so their biocompatibility had to be evaluated before they could be applied in a clinic setting. Many studies have shown that some materials show signs of toxicity when their diameters are reduced to nanoscale[14]. Therefore, the potential hazards and bio-safety of Fe_2O_3 microspheres should be particularly observed when they are applied to tissues. Biomaterials must not only have long-term stability in biotic conditions, but also have no harmful effects on tissues, blood or the immune system. In our work, referring to ISO10993-1992 and other international standards[15-17], we evaluated the nanoparticles using an *in vitro* cytotoxicity test, hemolytic test, a micronucleus experiment, by calculating the LD_{50}, and an *in vivo* study. MTT results showed that Fe_2O_3 nanoparticles had no significant effect on cellular proliferation when treated with various doses of extracted liquids of Fe_2O_3 nanoparticles. The cytotoxicities were 0 grade (RGR > 75%) indicating that there was no evidence of cytotoxicity. The results of the hemolytic test demonstrated that the hemolytic rate of the liquid-extracts of Fe_2O_3 and As_2O_3/Fe_2O_3 were 0.% and 0.77%, far less than 5%. This finding indicated that Fe_2O_3 had no hemolytic reaction when in direct contact with blood and was consistent with the requirement of hemolytic tests for biomaterials. Genotoxicity and

carcinogenicity tests answer the most complicated questions about biomaterials. The micronucleus assay is a rapid detection method to evaluate whether a biomaterial would damage chromosomes or interfere with cellular mitosis. This method can rapidly monitor acute and/or chronic genotoxicity, and does not require cultured cells[18]. In our study, we compared Fe_2O_3 groups with the negative control group and found no significant difference ($P > 0.05$) in the micronucleus formation rate. However, when we compared these groups with the positive control group, the result was significantly different ($P < 0.05$).

Therefore, Fe_2O_3 nanoparticles were not carcinogenic or mutagenic. However, the results of the acute toxicity test revealed that Fe_2O_3 nanoparticles intraperitoneally injected into the mice had low toxicity. The LD_{50} was equal to 5 g/kg, which is in the "no toxicity" category according to the standard of acute toxicity gradation of WHO. The LD_{50} of Fe_2O_3 for the mice was 5.75 g/kg with a 95% confidence interval of 4.8-6.9 g/kg. So, Fe_2O_3 also belonged to the "no toxicity" category and had a wide safety value margin. When we injected Fe_2O_3 into livers of healthy pigs, no significant differences in serum AST, ALT, BUN and Cr levels were found among pigs of all groups ($P > 0.05$), and no obvious pathological alterations were observed. From the results of our experiment, we believe that Fe_2O_3 demonstrated no toxic effects, is a highly biocompatible material and may be suitable for further applications in tumor hyperthermia.

We studied the therapeutic effect of Fe_2O_3 and As_2O_3/Fe_2O_3 combined with MFH on liver cancer *in vitro* and i*n vivo*. We injected Fe_2O_3 and As_2O_3/Fe_2O_3 into the tumor tissues instead of the normal tissue boundary of the tumor. Thus, the nanoparticles were delivered into the desired zone. This method allows thermogenic action to be administered locally, even in tumors located deep inside bodies, while minimizing heating of normal tissue around the tumor[19,20]. Compared with As_2O_3/Fe_2O_3 groups, As_2O_3/Fe_2O_3 combined with MFH had a better inhibitory effect on xenograft liver tumors, which indicates that MFH had a significant therapeutic effect. Much to our surprise, As_2O_3/Fe_2O_3 combined with MFH was the best therapeutic agent among all the groups tested. This result revealed that As_2O_3/Fe_2O_3 combined with MFH had two functions: chemotherapy of As_2O_3 and thermotherapy of magnetic Fe_2O_3 nanoparticles.

In conclusion, As_2O_3/Fe_2O_3 combined with MFH is a new biomaterial with low toxicity. However, we must acknowledge that our studies have limitations, and more researches should be carried out in the future. Although there is still a long way to go before the technology can be applied to clinical treatment, this method may develop into a new approach for the treatment of liver cancer and other solid tumors.

COMMENTS

Background

Hepatocellular carcinoma is one of the most common malignant tumors in China, and has a low recovery rate, so it is necessary to search for a new method to treat liver tumors.

Innovations and breakthroughs

Nanoparticles combined with magnetic fluid hyperthermia (MFH) have become a potential method to treat tumors. In this study, As_2O_3/Fe_2O_3 complexes were found to have two functions, chemotherapy of As_2O_3, and thermotherapy of magnetic Fe_2O_3 nanoparticles.

Applications

As_2O_3/Fe_2O_3 combined with MFH is a new biomaterial with good therapeutic effects on liver cancer. This preparation may be developed into a new agent for the treatment of liver cancer and other solid tumors.

Terminology

MFH is a method that can target and localize thermogenic actions. Therefore, the tissue surrounding the tumor without magnetic particles would not be damaged.

Peer review

The manuscript describes the therapeutic potential of nanosized As_2O_3/Fe_2O_3 complexes in combination with MFH on liver cancer cells. The authors analyzed the toxicity and therapeutic potentials of various concentrations of Fe_2O_3 and As_2O_3/Fe_2O_3. Interestingly, they found significant antitumor effects of those compounds against xenograft tumors of liver cancer cells when combined with magnetic fluid hyperthermia. The data are important and promising.

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