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The biocompatibility study of Fe_3O_4 magnetic nanoparticles used in tumor hyperthermia

Do not need to state non-membership.

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and

***Abstract*-To evaluate the biocompatibility of self-prepared Fe_3O_4 magnetic nanoparticles, which has the potential application in tumor hyperthermia, MTT assay was used to evaluate the in vitro citoxicity; hemolysis test was carried out to estimate its blood toxicity; Fe_3O_4 suspended in sterile 0.9%NaCl were intraperitoneally injected into KM mouse to calculate the LD_{50}; micronucleus (MN) were reckoned to identify its genotoxicity. The experiments showed that the toxicity of the material on mouse fibroblast (L-929) cell lines was between 0~1 degree;it has no hemolysis activity; Its LD_{50} arrived at 7.57g/kg; micronucleus test showed it has no genotoxic effects either. The results indicated that the self-prepared nanosized Fe_3O_4 is a kind of high biocompatibility materials and suitable for further application in tumor hyperthermia.**

BACKGROUND

Hyperthermia for tumor therapy has been long-standing. At present, thermotherapy commonly used in clinic such as radiofrequency, microwave, laster have much limitation in tumor hyperthermia for their respective shortcoming. In 1997, Jordan[1] discovered that nanoscaled magnetic fluid could absorbed the much higher power in alternating magnetic field ,which can be used for treatment of disease or tumor, named "Magnetic Fluid Hyperthermia(MFH)".MFH has a great virtue of targeting and localizing thermogenic action, therefore the tissue without magnetic particles would not be damaged. Fe_3O_4 magnetic nanoparticles (MNP) synthesized by an optimal co-prepcipitation technology have a gteat potentiality to be used in tumor MFH for their magnetoinduction thermogenic action. However the biomaterials would directly contact with the tissues and cells when they were sent into the body, so their biocompatibility had to be evaluated before they were applied in clinic[2,3]. Many studies showed that some materials would show signs of toxicity when their diameters go down to nano-scale[4], therefore, the potential hazards and bio-safety of Fe_3O_4 nanoparticles should be especially noticed when applied to tissue. In this work, the four aspects of Fe_3O_4 nanoparticles are first studied: *in vitro* cytotoxicity test, hemolytic test, the LD_{50} calculated, micronucleus experiment. Through the biocompatibility study of magnetic nanoparticles containing Fe_3O_4 by the above four experiments, it may be offered the credible academic-gists for future clinical application with Fe_3O_4 magnetic fluid hyperthermia.

MATERIALS AND METHODS

1. The extract-liquid of Fe_3O_4 MNP in vitro cytotoxity testing by MTT assay: The L-929 cells were cultured in RPMI1640 medium containing 10% fetal calf serum at 37°C in a 5%CO_2/95% air incubator with 95% humidity. The extract-liquid of Fe_3O_4 MNP were extracted using 1g of powder in 1mL complete RPMI-1640 medium for 72 h at 37°C. The cells were seeded in 96-well plate with 6000 cells per well, then the medium was replaced with 100%, 75%, 50%, 25% of extract liquid after 24h, the complete RPMI-1640 medium and 0.7% polyacrylamide monomer solution were provided as the negative control and the positive control respectively. The plates were continued to incubate for 48 h, then the MTT assay was performed and OD value was measured at 492nm. The cell relative growth rate (RGR) was calculated as following (OD of experimental group/OD of control group) ×100%.

2. Hemolytic test: Blood from healthy newzealand rabbit was collected in heparin-coated tubes. 1g of Fe_3O_4 MNP was placed in a tube containing 10mL 0.9%NaCl and suspended, 10mL 0.9%NaCl and 10mL of double distilled water were taken as negative (0% hemolysis) and positive (100% hemolysis) control, respectively. Each group contains three tubes. 0.2mL diluted anticoagulated cony blood were added to each tube which had been pre-heated at 37°C for 30min. Contents of all the tubes were incubated in a water bath at 37℃ for 1 h. Then all tubes were centrifuged at 2500 rpm for 5 min and supernatant was taken for the estimation of free hemoglobin. Absorbance was recorded at 545nm. The hemolysis rate (HR) was calculated as following: HR(%)=(OD of experimental group- OD of negative control group/ OD of positive control group- OD of negative control group)×100.

3. LD_{50} detection**:** The Kun Ming mice were divided into 8 groups randomly and in each group the quantity of female and male were both 5. Various weight of 100g/L Fe_3O_4 MNP were intraperitoneally injected into every mouse according to its weight, there are 7 experimental groups containing 1.77, 2.51, 3.54, 5.00, 7.06, 9.98 and 14.09g/kg. The negative control group was injected into the same volume of 0.9% NaCl, and the mice were observed in the following 15 days, then Median Lethal Dose (LD_{50}) was evaluated with Karber Method.

4. Micronucleus assay: the 60 mice (20-22g) were randomly divided into 6 groups and in every group the quantity of female and male were both 5. Animals were treated with Fe_3O_4 MNP (5g/kg, 3.75g/kg, 2.5g/kg, 1.25g/kg), negative group (with 0.9% NaCL) and positive group (with CTX 40mg/kg) were set as control groups. The mice were injected intraperitoneally 2 times (24 hours interval), and then at the sixth hour after the second injection, all the mice were killed.The thighbone marrows are extracted for smears, methanol fixed 5 minutes, then Giemsa dyed 15 minutes. Each smear counted 1000 polychromatic erythrocytes (PEC), and calculated the numbers of PEC containing micronucleus (MN). Poisson distribution verified each group of statistic difference.

RESULTS

There were many biomaterials applied in clinic in recent years. The biomaterials would directly contact with the tissues and cells while they were sent into the body. So, biomaterials had to have a kind of biocompatibility before they applied in clinic. In MTT assay, The RGR of L929 cells treated with 100%, 75%, 50%, 25% of extract-liquid of Fe_3O_4 MNP were 91.7%, 95.9%, 98.8%, 100.6% respectively(TABLE 1.), the results accorded with cells morphological changes by observing under inverted-microscope (Fig.1).It showed that it had no significantly effect to cellular proliferation treated with various doses of extract liquids of Fe_3O_4

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MNP, the cytotoxicity were 0~1 grade (RGR>75%) that belonged to no cytotoxicity. In hemolytic test, the HR of Fe_3O_4 MNP was 0.514% far less than 5%(TABLE 2.). So, we concluded that Fe_3O_4 MNP had no hemolytic reaction, and they consistent with the requirement of hemolytic test of biomaterials. The LD50 of Fe_3O_4 MNP to the mice was 7.57 g/kg and 95% confidence interval was 6.18~9.27 g/kg(TABLE 3.). So Fe_3O_4 MNP pertaining to no toxicity category actually and had widely safety value circumscription. In micronucleus assay, the MN formation rate of 5g/kg, 3.75g/kg, 2.5g/kg, 1.25g/kg experimental groups, negative control group and positive control group were 0.23%, 0.22%, 0.25%, 0.22%,0.20% and 24.8% respectively(TABLE 4.). Compared experimental groups with negative control group, we found that the result had no significantly difference (p>0.05) in micronucleus formation rate, while compared experimental group with positive control group, the result had significantly difference (p<0.05). So, it could believe that Fe_3O_4 MNP had no cacogenesis and mutagenesis. From the results of our experiment, we could consider that nanosized Fe_3O_4 (self preparation) had no toxicity basically, is a kind of high biocompatibility materials and perhaps is suitable for further application in tumor hyperthermia.

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Groups	Optical density (OD)	RGR/%	Cytotoxicity Gradations
Negative control group	0.6613±0.0324	100	0
25% extract liquid	0.6653±0.0310	100.6	0
50% extract liquid	0.6533±0.0364	98.8	1
75% extract liquid	0.6340±0.0371	95.9	1
100%extract liquid	0.6064±0.0435	91.7	1
Positive control group	0.1015±0.0063	15.3	4

TABLE 1. The results of MTT test.

Groups	Optical density (OD)			Average OD	Hemolysis rate(HR)/%
	1	2	3		
Negative control group	0.017	0.014	0.019	0.0167	
MNP extract group (t)	0.018	0.021	0.023	0.0207	0.514
positive control group	0.776	0.768	0.789	0.7777	

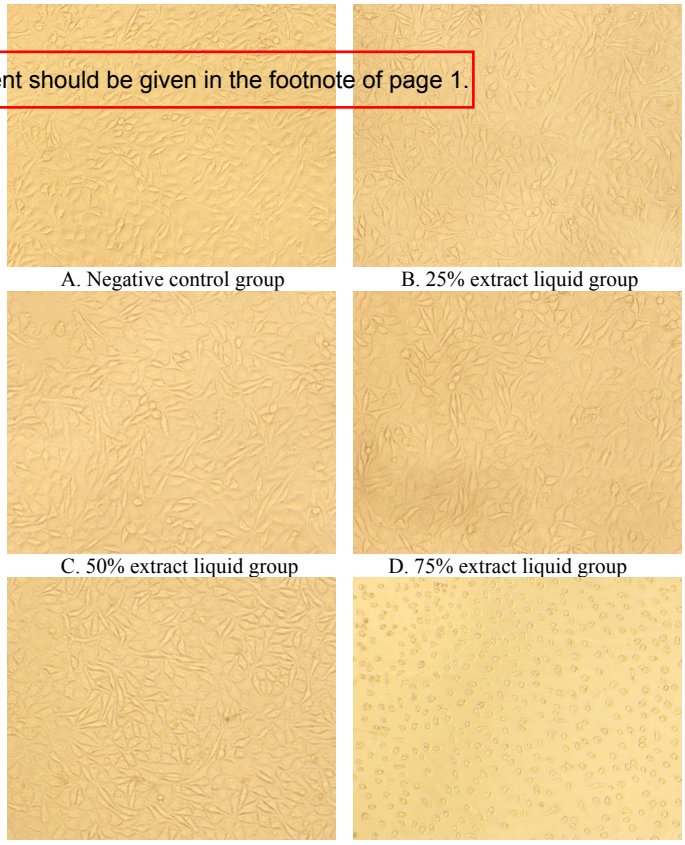
TABLE 2. The results of hemolytic test of Fe_3O_4 magnetic nanoparticles extract liquid

Groups	Dosages		Mice numbers (n)	Death numbers	Mortality (p)	Livability (q)	p×q
	g/Kg	Logarithm					
1	1.77	0.249	10	0	0	1.0	0
2	2.51	0.399	10	0	0	1.0	0
3	3.54	0.549	10	1	0.1	0.9	0.09
4	5.00	0.699	10	3	0.3	0.7	0.21
5	7.06	0.849	10	5	0.5	0.5	0.25
6	9.98	0.999	10	5	0.5	0.5	0.25
7	14.09	1.149	10	9	0.9	0.1	0.09
i=0.15						∑p=2.3	

TABLE 3. The results of acute toxicity test of Fe_3O_4 magnetic nanoparticles.
$lgLD_{50}=X_k-I(\sum p-0.5)= 1.149-0.15(2.3-0.5)=0.879$
$s_m=i\times(\sum pq/n)^{1/2}=0.15\times0.2983=0.045$
MNP lg LD_{50} and its 95% confidence limit : $0.879\pm1.96\times0.045=0.879\pm0.088$
$MNPLD_{50}$ and its 95% confidence interval: 7.57g/kg (6.18～9.27 g/kg)

Groups	Numbers (n)	PEC numbers	PEC numbers containing MN	MN-formation rate(%)
Negative control	10	10000	22	0.22
5.00g/kg	10	10000	23	0.23[1)]
3.75g/kg	10	10000	20	0.20[1)]
2.50g/kg	10	10000	24	0.24[1)]
1.25g/kg	10	10000	22	0.22[1)]
Positive control	10	10000	253	2.53[2)]

TABLE 4. The results of micronucleus assay, Compare experimental group with negative group, [1)]P>0.05; Compare positive group with negative group, [2)]P<0.05.



A. Negative control group B. 25% extract liquid group
C. 50% extract liquid group D. 75% extract liquid group
E. 100% extract liquid group F. Positive control group

Fig. 1. The inverted microscopic pictures of L929 cells which treated by the extract liquid of Fe_3O_4 nanoparticles in MTT test(×100).