In vitro & in vivo toxicity of $CoFe_2O_4$ for application to magnetic hyperthermia

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ABSTRACT

Magnetic nanoparticles offer some attractive possibilities in biomedicine because it has the special physical properties. Magnetic hyperthermia using magnetic nanoparticles requires that the magnetic particles have not only high heating ability in low magnetic field but also to be non-toxic for biomedical use. Although the reports of bulk magnetic materials or Fe_3O_4 nanoparticles have known for hyperthermia, it has not reported about application to hyperthermia and the biological responses of $CoFe_2O_4$ nanoparticles. Therefore, we investigated the *in vitro & in vivo* toxicity as well as heating ability under magnetic field for application of $CoFe_2O_4$ nanoparticles to hyperthermia.

Keywords: $CoFe_2O_4$, magnetic nanoparticles, toxicity, hyperthermia

1 INTRODUCTION

Nanomaterials are receiving increasing attention for their promise as engineering and biomedical miracles. In fact, nanomaterials help in overcoming the limitations of size and can change the outlook of the world regarding science. For examples, magnetic nanoparticles in gene delivery, biosensors, drug delivery and hyperthermia had attracted increasing fundamental and technological interests. However, their use may ultimately be limited, because the lack of information regarding the toxicity of synthesized magnetic nanoparticles poses serious problems [1]. Therefore, the toxicity of magnetic nanoparticles has raised a critical factor on evaluating their applications, recently.

Hyperthermia using magnetic particles has been recognized as a useful therapeutic modality in the treatment of malignant tumors. The magnetic nanoparticles with alternating magnetic field are expected useful thermoseed in hyperthermic cancer treatment since they can be targeted and confined to the cancer site without damaging normal cell. However, for the successful bio-application of magnetic nanoparticles in hyperthermia, it is essential to understand the biological fate and potential toxicity of nanoparticles. The production of localized hyperthermia for tumor treatment has been hindered by a number of physical and biologic factors. Therefore, the magnetic nanoparticles for magnetic hyperthermia require not only optimistic magnetic properties and narrow particle size distribution but also to be non-toxic and biocompatibility. The magnetic nanoparticles are completely foreign to the biological environment; therefore it is important that they must be tested for any harmful effects on the cells. In this study, we focused the magnetic hyperthermia using $CoFe_2O_4$ nanoparticles among various biomedical applications. We had studied $CoFe_2O_4$ nanoparticles for high effective hyperthermic thermoseed [2]. Iron oxide particles already known as nontoxic magnetic materials and also MRI contrast agents have already been approved for human use [3]. However, it was not reported about the toxicity of $CoFe_2O_4$ in hyperthermia or biomedical uses in detail. Therefore, we investigated the biocompatibility as well as the heating ability of $CoFe_2O_4$ particles *in vitro* and *in vivo*.

2 MATERIALS AND METHODS

$CoFe_2O_4$ nanoparticles were synthesized by sol-gel route. Their magnetic and structural properties were determined by XRD, VSM and SEM, respectively. The exothermic energy from the $CoFe_2O_4$ suspensions was monitored for 300 sec under alternating magnetic field (150 KHz, 7kW). The cytotoxicity in various doses $CoFe_2O_4$ was examined using the MTT assay. The acute systematic toxicity in vivo test was investigated with ten of ICR mice. Animals were checked clinical signs and weight of animals were also measured for 7 weeks after injection. And histological analysis was performed on stained organs with Haematoxylin and Eosin or Prussian blue.

2.1 Preparation and Characterization

Cobalt acetate tetrahydrate [$Co(CH_3CO_2)_2 \cdot 4H_2O$], and iron nitrate nonahydrate [$Fe(NO_3)_3 \cdot 9H_2O$] were selected as precursors of $CoFe_2O_4$ nanoparticles. They were dissolved in acetic acid, methanol and water for 1 h. This solution was refluxed at room temperature for 24 h to allow gel formation and dried at 100℃ for 24 h. Then, it was fired at 800 ℃ for 6 h in air. The structures of samples were determined by X-ray diffraction (XRD; D/MAX Rint2000,

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Rigaku, Japan) using Ni-filtered Cu-kα X-rays with the phase being identified by a comparison with the JCPDS database. A vibrating sample magnetometer (VSM7300, Lakeshore, USA) was used to measure the saturation magnetization (Ms) and the coercive force (Hc) of each sample. The average size, size distribution and morphology were estimated using transmission electron microscopy (JEM4010, JEOL, Japan). An external alternating magnetic field was applied to the magnetic particles using a heating system (SPM7S, YoungChang, Korea). The exothermic energy from the samples was monitored by a thermometer [alcohol] under an alternating magnetic field (frequency: 150 KHz, 3kW). The SAR (specific absorption rate) of the samples was determined by time-dependent temperature measurements. The concentration of all samples was 10 mg/ml in phosphate buffered solution (PBS; Gibco, USA) and treated with ultrasound for 10 min before the measurement.

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2.2 *In vitro* cell viability/cytotoxicity studies

The established L929 cell line (fibroblast connective tissue) from the Korean Cell Line Bank (KCLB, Korea) was used in these experiments. The cytotoxicity of cobalt ferrite suspensions in different concentrations was examined using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method. The MTT assay is a simple non-radioactive colorimetric assay to investigate the level of cell cytotoxicity, proliferation or viability through measuring the succinate dehydrogenase mitochondrial activity. MTT is a yellow, water-soluble, tetrazolium salt. Metabolically active cells can convert this dye into a water-insoluble dark blue formazan by the reductive cleavage of the tetrazolium ring [4]. Formazan crystals can then be dissolved in an organic solvent such as dimethylsulphoxide (DMSO) and quantified by measuring the absorbance of the solution at 570 nm, and the resultant value is related to the number of living cells. In order to determine the cell cytotoxicity/viability, the cells were plated into 96 well plates at a density of 10,000 cells/well in RPMI supplemented with 10% fetal bovine serum (FBS). at 37℃ in a 5% CO_2 atmosphere. After 24 h incubation, the medium was replaced with fresh medium and 0.02 ml of particle suspension with different concentration was added to each well. As controls, cells were treated with an equivalent volume of PBS without any particles. Different concentrations of particles added 0.05wt% polyvinyl alcohol (PVA) for surfactant were suspended in PBS solution using ultrasonication for 10 min to prevent agglomeration [5]. After 24 h, 0.05 ml of a MTT dye solution (5 mg/ml in phosphate buffer pH-7.4) was added to each well. After 4 h incubation at 37℃ in 5% CO_2 to exponentially grow the cells, and 15 min to produce steady-state confluent cells, the medium was removed and the formazan crystals were dissolved in 0.05 ml of DMSO and the solution was mixed vigorously to dissolve the reacted dye. The absorbance of each well was read on a microplate reader (MRX microplate reader, DYNATECH, USA) at 570 nm. The spectrophotometer was calibrated to zero absorbance using a culture medium without the cells. The relative cell viability (%) related to the control wells containing the cell culture medium without the magnetic particles suspension was calculated using the following formula: $[A]_{test}/[A]_{control}X100$.

2.3 *In vivo* Acute systematic toxicity test (Intravenous injection)

Animal selection and management, surgical protocol, and preparation followed routines approved by the Institutional Animal Care and Use Committee, Yonsei Medical Center, Seoul, Korea. For the acute systematic test, ICR mice, 6 weeks of age weighing 22 ~ 25 g, were used. Ten male ICR mice were assigned for each test groups. All test procedure was followed by ISO10993-11. We selected 1 ml of $CoFe_2O_4$ particle suspension (10 mg/ml). The sample was performed by autoclaving 121 ℃ for 1 hour. Each mouse received a single intravenous injection of magnetic suspension by 24 gauge needle syringe for 5 min. Animals were checked clinical signs such as diarrhea, piloerection, and mortality and body weight of animals were also measured for 7 weeks after injection. For the histological examination, test animals were anesthetized after acute systematic toxicity test and vital internal organs of each mouse were excised. Samples of these organs were fixed in 8% formalin, embedded in paraffin wax, sectioned at 5 ㎛ and stained with haematoxylin and eosin (H&E). For the detection of iron particles, Prussian blue was stained with section of each organ. Detailed microscopic examination was carried out on organs of animals.

3 RESULTS

$CoFe_2O_4$ nanoparticles were synthesized by sol-gel process. Their magnetic and structural properties of the particles were determined using an x-ray diffractometer and vibrating sample magnetometer, respectively.

	Particle size(nm)	Ms (emu/g)	Hc (kOe)	Crystal structure	Δ T
$CoFe_2O_4$	68	72	23.9	Spinel	23.5℃

Table 1: Characterization of prepared sample.

The XRD diffraction patterns of the phase evolution of the $CoFe_2O_4$ nanoparticles exhibit a spinel structure (Table 1). The magnetic properties of the prepared $CoFe_2O_4$ nanoparticles showed the saturation magnetization, 72 emu/g and the coercivity, 23.9 kOe. The temperature change of the 5 ml of $CoFe_2O_4$ suspension (10 mg/ml) under alternating magnetic field (150 kHz and 3 kW) was ΔT=23.5 ℃ for 10 min. And the saturation of increasing rate in temperature change was represented at 46 ℃ after 8 min, approximately.

Cell viability of $CoFe_2O_4$ nanoparticles are shown in Fig. 1. There was no decrease of cell viability as increasing the concentration of $CoFe_2O_4$ nanoparticles suspension. The cytotoxicity of $CoFe_2O_4$ was not seen at concentration below 10 mg/ml. The cell viability at concentration of ≤ 10 mg/ml was 100-76% which is mild toxicity. However, the cell viability of $CoFe_2O_4$ magnetic suspension was 48.2 % at the concentration of 2000 μg/ml.

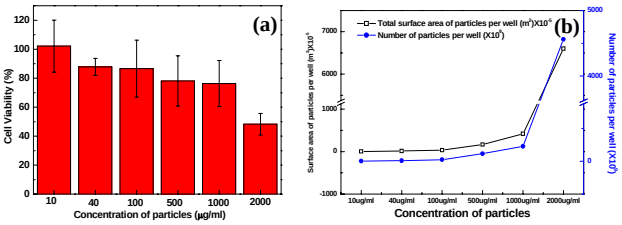

Figure 1 : (a) Cell viability of $CoFe_2O_4$ nanoparticle in different concentration (10, 40, 100, 500, 1000 and 2000 μg/ml) and (b) Total surface area and number per well according to the concentration of $CoFe_2O_4$ nanoparticles.

The cytotoxicity of $CoFe_2O_4$ nanopartices was determined by the number and the surface area because cytotoxicity is represented by exposure of cells to toxicants. Therefore, the number and surface area showed in Fig. 2. The large surface area of $CoFe_2O_4$ nanoparticles increases a contact with cell layer. Cell damages will be affected by surface area rather than the concentration of particles [6]. Cell reproduction can be measured by several parameters including cell count, DNA content, protein content, or enzyme activity. Although a morphological alteration can't observe in the cell layer and/or cell shape, MTT assay in this study is a simple non-radioactive colorimetric assay to evaluate the level of cell cytotoxicity, proliferation or viability through measuring the dehydrogenase mitochondrial activity.

As preliminary study of hyperthermic treatment in vivo, the acceptable amount of $CoFe_2O_4$ nanoparticles suspension in vivo should be determined with injecting the $CoFe_2O_4$ nanoparticles suspension. Thus, the amount of injecting $CoFe_2O_4$ nanoparticles suspension was determined by the in vitro test and other references [7]. The 1 ml of $CoFe_2O_4$ particle suspension (10 mg/ml) was selected for *in vivo* toxicity test. *In vivo* toxicity test, there was no significant difference in body weight compared to control group in Fig. 2. During the study period, injected with $CoFe_2O_4$ nanoparticls suspension for 7 weeks did not cause any adverse effects on growth. In addition, abnormal clinical signs or behaviors were not detected in both groups. In histopathologic evaluation, Fig. 3 showed that $CoFe_2O_4$ nanoparticles are distributed in each organ. As shown in Fig. 3, $CoFe_2O_4$ nanoparticles were found in almost all organs such as heart, kidney, liver, spleen and stomach [8]. The highest amount of $CoFe_2O_4$ nanoparticles were taken up by the liver, but not seen in the lung. The reticuloendothelial system (RES) is the liver, spleen and lung and non-RES organs is heart, kindeny and stomach. In our study, the localization of $CoFe_2O_4$ nanoparticles was not consistent with the RES system. And most of the intravenously injected particles are recognized by the body as being the foreign and subsequently removed from the blood circulation by the macrophages of the mononuclear phagocytic system (MPS). This so called "MPS clearance" leads to a high uptake of particles in the Kupffer cells of the liver, and about 80-90% of the injected dose accumulate in this organ within 5 minutes after injection. A lower percentage of about 2-5% is taken up by the macrophages of the spleen, and only little is found in other organs such as lungs, kidneys, heart and bone marrow [9]. These are well matched with our results. The $CoFe_2O_4$ nanoparticles might give more damages to liver in over dose than other organs. Fig. 4 showed light micrographs for histological evaluation of the organs from the mice injected $CoFe_2O_4$ nanoparticles suspension. From the Fig. 4, abnormal histopathological findings were not observed. The liver from $CoFe_2O_4$ injected mouse reveals no remarkable changes in the dose of 20 mg $CoFe_2O_4$ nanoparticles, such as cytoplasmic vacuolization and karyorrhexis. And, no atypical tubules and necrosis were also observed in kidney of test group.

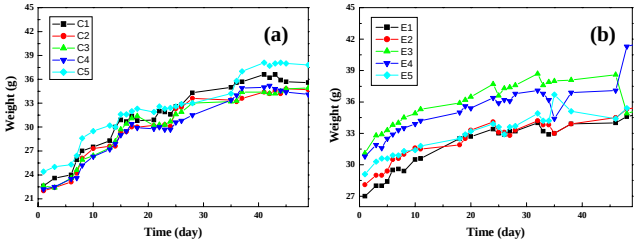

Figure 2 : Weight changes of (a) control and (b) $CoFe_2O_4$ injected mice group for acute systemic toxicity.

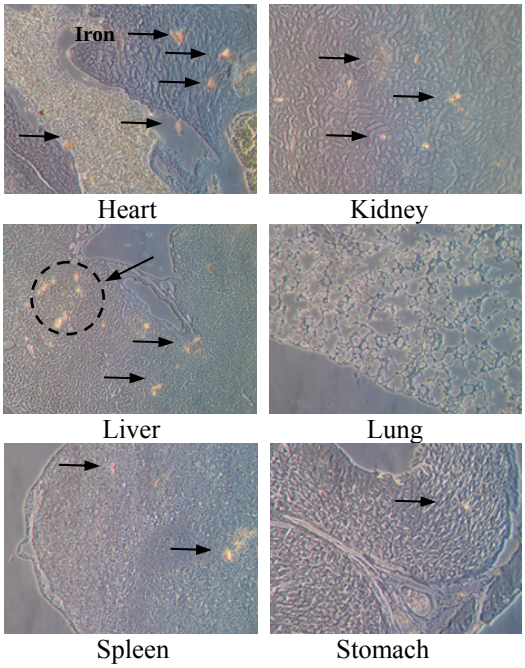

Figure 3 : Light microscope images of tissue from test group (Prussian Blue staining).

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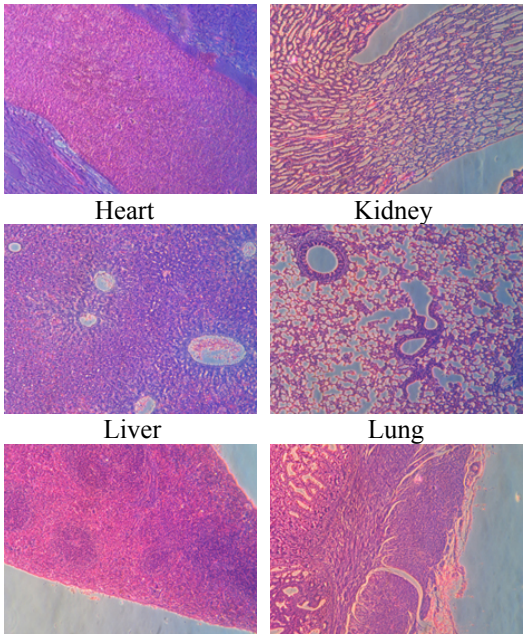

Figure 4 : Light microscope images of tissue from test group (H&E staining).

4 CONCLUSION

The biologic factors were important for clinical application. The important factors, which determine the biocompatibility and toxicity of ferrite, are the magnetically responsive components such as magnetite, iron, nickel-zinc-copper, lithium, nickel, cobalt ferrite [10]. In order to minimize cytotoxic problems in the body before biomedical applications, the materials must be assessed for compatibility with biological environment. $CoFe_2O_4$ nanoparticles were synthesized by sol-gel route. The temperature change under alternating magnetic field and saturation magnetization (Ms) was ΔT=23.5 ℃, Ms=72 emu/g. The cell viability at concentration of ≤ 10 mg/ml was 100-76% which is mild toxicity. The cytotoxicity of $CoFe_2O_4$ nanopartices would be determined by the number and the surface area because the cytotoxicity is raised by exposure of cell layer to toxicants. Therefore, cell damages will be affected by surface area rather than the concentration of particles. *In vivo* toxicity test, during the study period, injected with $CoFe_2O_4$ nanoparticls suspension for 7 weeks did not cause any adverse effects on growth because no significant differences in the body weight gain or loss between control and test group. In addition, abnormal clinical signs or behaviors were not detected in both groups. In histopathologic evaluation, $CoFe_2O_4$ nanoparticles were found in almost all organs such as heart, kidney, liver, spleen and stomach. The tissue images of the $CoFe_2O_4$ injected group reveals no remarkable changes compared with control. Finally, $CoFe_2O_4$ could be expected as bio-materials for magnetic hyperthermia through the results of heat generation and *in vitro* & *in vivo* toxicity test.

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