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ORIGINAL ARTICLE

Toxicity Assessment of DMSA Coated and Noncoated Iron Oxide Nanoparticles in Mice

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ABSTRACT
In this study, the effect of DMSA as the biocompatible material on the surface of Fe_3O_4nanoparticleswas compared with noncoated nanoparticles. The properties of nanoparticles were evaluated by AGFM, TEM and XRD. The chemical interaction between Fe_3O_4 and DMSA was investigated by FTIR. The effect of single IP (100mg/kg) of these nanoparticles was studied in liver and kidney,2, 10 and 30 days after treatment. Noncoated nanoparticles did not any changes in the liver function compared to the control group. SGPT and LDH enzymes were not changed significantly in DMSA coated nanoparticles. But, ALP was increased. Also, the SGOT increase significantly 30 days after treatment. The uric acid was reduced after the noncoated nanoparticles injection whereas it has changed in DMSA coated injection. The urea was increased in DMSA coated injection. The mice weight was not changed during treatment. Presence of DMSA on the surface of iron oxide nanoparticles probably increases their biocompatibility in blood and tissue. This phenomenon consequently causes transient impairment in liver and kidney function. Histological studies didn't show any disorder in liver and kidney tissue. Therefore, Fe_3O_4 nanoparticles, even with DMSA coat have not any serious health risk or toxicity on the body in mice.
Keywords: Biocompatible, Superparamagnetic nanoparticles, Liver, Kidney.

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INTRODUCTION

Magnetic nanoparticles have been used for different medical applications [1,2]. There are many different methods for synthesizing magnetic nanoparticles such as gamma-irradiation [3], electron beam radiation [4], low-temperature solid state reaction[5],co-precipitation[6]and microwave method [5]. Nowadays, nanoparticles are coated with biocompatible materials such as PEG (polyethylene glycol, $C_{2n}H_{4n+2}O_{n+1}$), polyaspartic acid,dextran and DMSA (dimercaptosuccinic acid $C_4S_2O_4H_6$) in order to increase their stability in the biological solutions and blood circulation and also to decrease their toxicity effects. In this middle, magnetic nanoparticles which coated with organic molecules can create stable colloids (biocompatible magnetic fluids) in physiological conditions[7,8,9,10].

Iron oxide (Fe_3O_4) is one of the most important superparamagnetic nanoparticles. However, there are few reports on its toxicity or side effects on cell and fauna, especially under in vivo conditions. Moreover, limited researches conducted on this topic have had controversial results. For example, some studies have reported non-toxicity of iron oxide nanoparticles under in vivo conditions and some others have reported minimal toxicity or an inflammatory response and severe cell death[8,11,12,13,14,15,16].

On the other hand, considering the variety of coatings used on the surface of nanoparticles makes it necessary to separately study the tissue distribution and side effects of iron oxide nanostructures with any particular coating in order to find out appropriate coverage for different biological and medical cases[8,13,15]. In this research, toxic effects of DMSA-coated and noncoated iron oxide nanoparticles were investigated in both of kidney and liver factors of the mice.

MATERIALS AND METHODS

Method of producing iron oxide nanoparticles and quality assurance

In order to produce DMSA coated iron oxide nanostructure (Fe_3O_4 @ DMSA), the wet chemical method was used which is well described in previous works [17]. First of all,the solutions of $FeCl_2$(0.01 M), $FeCl_3$ (0.02 M) and NaOH (0.08 M) were prepared in distilled and deionized water (all water from Merck Company). In the construction process, DMSA (0.01M) was added to the solution.

The size and structure properties of Fe_3O_4@DMSA nanoparticles were assessed by TEM (Transmission Electron Microscopy, LEO-912AB) and XRD (X-Ray Diffraction, Bruker D8 ADVANCE λ=0.154nm Cu Kα radiation, Germany). The chemical interaction between Fe_3O_4 and DMSA was investigated by FTIR (Fourier Transform Infrared Spectroscopy, Bruker TENSOR 27, Germany). Magnetic properties of Fe_3O_4@DMSA nanoparticles were investigated by AGFM (Alternating Gradient-Force Magnetometer, Meghnatis Daghigh Kavir Co, Iran) [17,18].

Breeding animals and treatments

For this purpose, 45 female mice of Balb/C strain were prepared from RAZI Vaccine and Serum Research Institute. They were 3-months old and were kept in natural light and humid at 22-24 ºC temperature. They were divided into three equal groups (each group contains 15 mice). One group was injected with uncoated Fe_3O_4 nanoparticles and the other group was received Fe_3O_4 @ DMSA. The last group which is the control group just was injected with normal saline. All of the injections were IP (Intra-peritoneally) and the injected dose was 100mg iron oxide per one kg of mice weight (100mg/kg). The animal studies were performed in accordance with regulatory guidance on the care and use of experimental animals.Mice's weight was measured and recorded at the time of injection and 2, 10 and 30 days postinjection.

Measurement of kidney and liver factors

Blood samples were taken directly from the heart under mild anaesthesia with ether, from all groups 2 (treatment 1), 10 (treatment 2) and 30 days (treatment 3) post-injection (five mice from each group in every time interval). Samples were poured into the special pipes which contain EthyleneDiamine Tetraacetic acid (EDTA) anticoagulation agent. After that, kidney and liver factors such as lactate dehydrogenase (LDH), serum glutamic pyruvate transaminase (SGPT), serum glutamic oxaloacetic transaminase (SGOT), alkaline phosphatase (ALP), Albumin protein, uric acid, creatinine, and urea were measured by Hitachi Automatic Analyzer (Roche 902, Japan).

Tissue section preparation for hematoxylin and eosin staining

For this purpose, several mice were dissected in every treatment groups immediately after bleeding. Kidney and liver tissue sections were prepared because kidneys are responsible for drug exclusion from blood circulation and the liver is the most important organ for drug metabolism. Tissue sections were stained to observe nanoparticles accumulation using specific iron Prussian blue method (Hematoxylin and eosin staining). Accumulation of Fe_3O_4 nanoparticles was demonstrated as dark blue grains in the tissue sections using light microscope.

Statistical analysis

The mean values of kidney and liver factors (with group segregation) and also mice's weight (with time segregation) were compared by ANOVA test (analysis of variance) and t-test by SPSS (version 15) computer program in all groups. Results are the mean of five separate experiments for each group.

RESULTS

The results of TEM, XRD, FTIR and AGFM

Figure 1 indicates the TEM photograph of the Fe_3O_4@DMSA nanoparticles.As can be seen, the size of the particles is around 10 to 15 nm with approximately uniform size distribution. The XRD pattern of both Fe_3O_4 andFe_3O_4@DMSA nanoparticles is demonstrated in figure 2.The mean particle diameter forFe_3O_4@DMSA nanoparticles were also determined around 12 nm from XRD pattern by Debye-Scherer formula which is consistent with the TEM result (calculations are not shown here). From XRD patterns, it also results that both of Fe_3O_4 andFe_3O_4@DMSA nanoparticles are single phase and have spinel structure.

The chemical interaction between Fe3O4 and DMSA were investigated by FTIR and it resulted that the coating interaction has been done successfully (FTIR curve is not shown here and can be found in previous work) [17].

The hysteresis loop of Fe_3O_4 and Fe_3O_4@DMSA nanoparticles obtained by AGFM and there is no remanent in both curves. Therefore, both of them have the super paramagnetic property.The saturation magnetization was measured as 62 emu/g for Fe3O4 whereas it was determined as 27 emu/g for Fe3O4@DMSA (as shown elsewhere) [17,18].

The effect of noncoated Fe_3O_4 nanoparticles on the kidney and liver parameters

Table 1 indicates the mean values of kidney and liver parameters in 2 (treatment 1), 10 (treatment 2) and 30 days (treatment 3) post-injection (uncoated nanoparticles). As can be seen, the mean values of liver parameters (except albumin) don't show any significant difference between all groups. The mean albumin value (3.79 ± 0.36) decreased in the second treatment group (10 days after treatment)but it returned to the normal value 30 days post-injection.The fluctuations in mean values of AlP, LDH,SGOT and SGPT were not significant for all groups and returned to normal 30 days post-injection. Among the kidney factors, there is a meaningful difference in the uric acid mean value (0.52 ± 0.06) 10 days post-injection with that of normal value. It didn't return to normal even 30 days post-injection (0.47 ± 0.09). The rest of kidney factors don't show any meaningful difference in comparison with the normal value.

The effect of DMSA-coated Fe_3O_4 nanoparticles on the kidney and liver parameters

The mean values of kidney and liver parameters in 2 (treatment 1), 10 (treatment 2) and 30 days after the injection of DMSA coated Fe_3O_4 nanoparticles are shown in Table 2. The significant increase in AlP means value (42.7±8.5) is seen 2 days post-injection (treatment 1). This change is returned to normal in second and third treatment groups. Also, the significant differences are observed in SGOT mean value 10 (304.3±28.9) and 30 days (253.9±26.6) post injection. The uric acid mean value is increased in the first treatment group (1.2±0.22) but returned to normal in other treatment groups. Whereas it significantly decreased 10 days after the noncoated nanoparticles injection and it didn't return to normal up to one month. Moreover, the urea mean value is significantly increased (92.8±6.9) 30 days post-injection (treatment 3)whereas; it didn't happen for that of noncoated nanoparticles injection.

On the other hand, other kidneys and liver parameters don't show any meaningful changes in all groups compared with that of the control group. Also, there is not any significant change in mice's weight in all groups (Results are not shown here).

Stained kidney and liver tissue sections observation

Figure 3, indicates the stained liver tissue sections 30 days post-injection for coated and noncoated nanoparticles in comparison with control. As can be seen, there is not any tissue disorder or disturbance in the hepatocytes, sinusoids and centriolobular venule. Also, neutrophil count and kuffer cells accumulation are normal and therefore there is not any tissue inflammation.

Figure 4, demonstrates the same photo from the kidney tissue sections.It can be seen that there is not any disorder in the glomerulus,proximal convoluted tubules and distal convoluted tubules. Moreover, high neutrophils and eosinophils accumulation were not seen and therefore there is not any inflammation response.

Table 1. Mean values of kidney and liver parameters in different time intervals post injection (Single injection of noncoated iron oxide nanoparticles, 100mg/kg)

Groups (n=10)	Liver parameters					Kidney parameters		
	AlP (U/L)	LDH (U/L)	SGOT (U/L)	SGPT (U/L)	Alb (g/dl)	Urea (mg/dl)	Uric Acid (mg/dl)	Creatinine (mg/dl)
Control	17±4.2	1395±50.4	181±22.6	52±5.9	4.34±0.26	60.8±7.2	0.80±0.14	0.35±0.08
Treatment	11±3.9	1154±94.5	185±26.9	51±7.0	4.17±0.24	52.7±9.7	1.05±0.16	0.30±0.08
Treatment	6.5±1.9	1069±77.7	154±22.0	64±7.7	3.79±0.36 *	67.7±7.4	0.52±0.06 ®	0.33±0.03
Treatment	15±3.9	1453±106.0	208±20.5	54±9.0	4.37±0.38	53.4±8.7	0.47±0.09 ®	0.36±0.04

*The significant difference (P<0.05, F=3.24, ANOVA)

® The significant difference (P<0.01, F=7.34, ANOVA)

All values are the mean of 5 separate measurements.

Table 2. Mean values of kidney and liver parameters in different time intervals post injection (Single injection of DMSA coated iron oxide nanoparticles, 100mg/kg)

Groups (n=10)	Liver parameters					Kidney parameters		
	AlP (U/L)	LDH (U/L)	SGOT (U/L)	SGPT (U/L)	Alb (g/dl)	Urea (mg/dl)	Uric Acid (mg/dl)	Creatinine (mg/dl)
Control	17±4.2	1395±50.4	181±22.6	52±5.9	4.34±0.26	60.8±7.2	0.80±0.14	0.35±0.08
Treatment 1	42.7±8.5 *	1159.9±88.5	201.5±29.7	54.5±8.8	4.21±0.64	66.2±14.4	1.2±0.22 ®	0.32±0.02
Treatment 2	20.2±4.0	1248.7±96.1	304.3±28.9 ª	63.9±9.5	4.06±0.53	61.7±4.1	0.62±0.25	0.35±0.03
Treatment 3	28.8±4.6	1215.9±88.4	253.9±26.6 ª	52.8±9.2	4.00±0.55	92.8±6.9 β	0.66±0.09	0.40±0.04

* The significant difference (P<0.01, F=7.17, ANOVA)

ª The significant difference (P<0.01, F=12.43, ANOVA)

β The significant difference (P<0.01, F=27.84, ANOVA)

®The significant difference (P<0.01, F=7.12, ANOVA)

In conclusion, in vivo application of low concentrations of iron oxide nanoparticles (less than 100 mg/kg) has not probably any serious toxic effects in biological systems. Moreover, there was not any meaningful changes in mice's weight during a month post-injection. According to the controversial findings in different studies, it seems that more investigations with appropriate biological coatings are necessary.

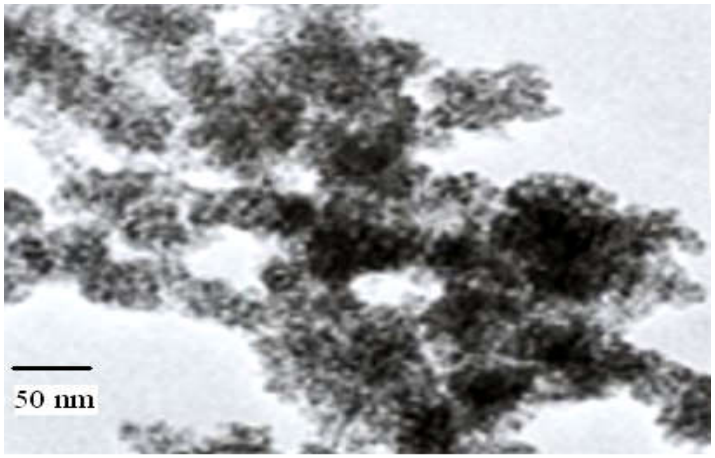

Figure 1. TEM photograph of the Fe_3O_4@DMSA nanoparticles.

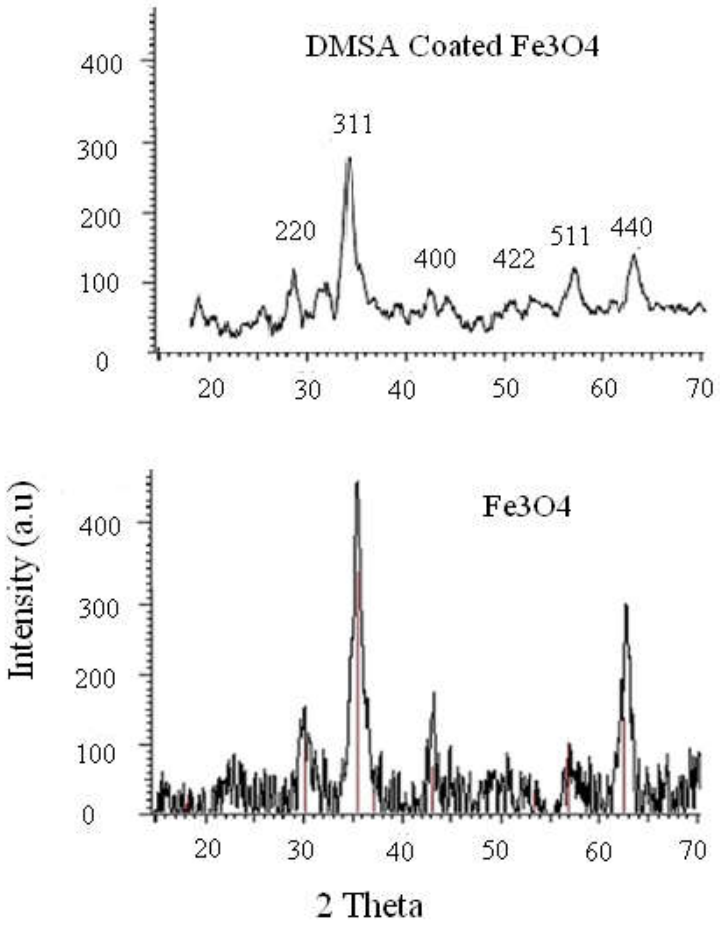

Figure 2. XRD pattern of Fe_3O_4 and Fe_3O_4@DMSA nanoparticles.

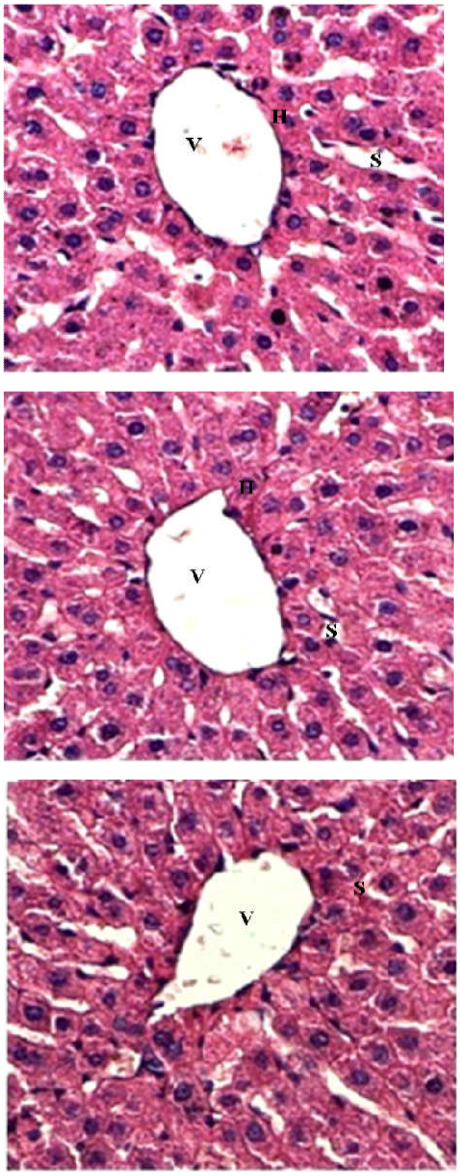

Figure 3. Mice liver tissue section 30 days post-injection (Hematoxylin and eosin staining, magnification 40x) A. DMSA coated Fe_3O_4 nanoparticle, B. Noncoated Fe_3O_4 nanoparticle, C. Control
H= hepatocyte, V= centriolobular venule, S= sinusoid.

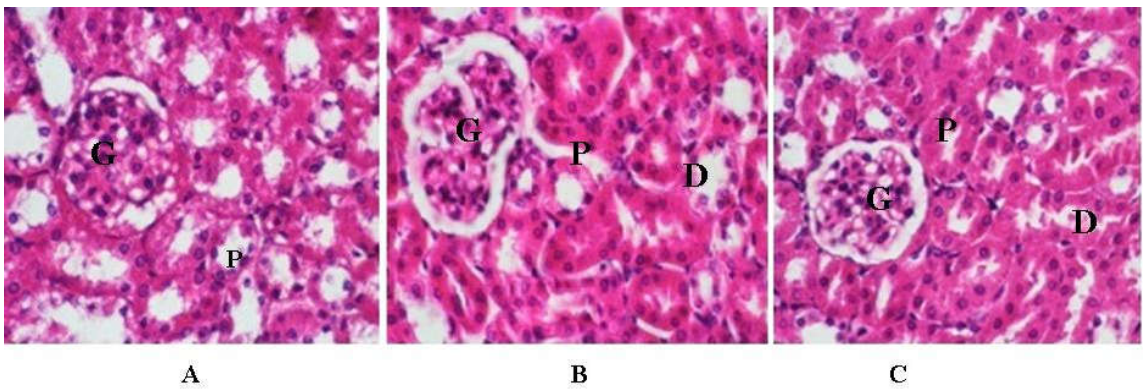

Figure 4. Mice kidney tissue section 30 days post-injection (Hematoxylin and eosin staining, magnification 40x) A. DMSA coated Fe_3O_4 nanoparticle, B. Noncoated Fe_3O_4 nanoparticle, C. Control
G= glomerulus, P= proximal convoluted tubules, D= distal convoluted tubules.

DISCUSSION

The results of XRD, AGFM and FTIR (Figures 1 and 2) indicated spinel structure, crystallite size and magnetic properties of Fe3O4 nanoparticles with and without DMSA that correspond with previous studies[19].

In this study, the saturation magnetization was measured as 62 emu/g for Fe3O4 whereas it was determined as 27 emu/g for Fe3O4@DMSA (as shown elsewhere) [20]. This magnetization reduction is due to the presence of nonmagnetic DMSA molecules on the surface of Fe3O4. Moreover, since the critical size for Fe3O4 nanoparticles is 30 nm and according to the TEM and XRD results, size of the nanoparticles were obtained much less than 30 nm, these particles are expected to be superparamagnetic which has a good agreement with AGFM results.

The effects of intra-peritoneally injection of DMSA coated and noncoated iron oxide nanoparticles (100 mg/kg) on mice's kidney and liver function were investigated. The results showed that some kidney factors and liver enzymes were changed significantly. However, this situation was temporary and most of them returned to their normal range after a month. Also, no difference was observed in liver and kidney tissue sections.

The findings seem to prove the fact that DMSA-coated Fe_3O_4 nanoparticles are more effective in comparison with noncoated Fe_3O_4 nanoparticles. This phenomenon happened probably due to their more stability in blood circulation and consequently more penetration in different organs and cells. Therefore, it seems that the application of DMSA as a biological coat increases the nanoparticles stability and their side effects on kidney and liver function.The findings show that the intraperitoneal injection of 100mg/kg of noncoated iron oxide nanoparticles has not any serious toxicity effect on the mice's liver and kidney function.

This result is similar to that of Kim *et al*, which they intra-peritoneally injected 25, 50 and 100 mg/kg concentrations of magnetic nanoparticles ($CoFe_2O_4$) coated with silica (SiO_2) into mice and observed their presence and distribution in organs such as liver, spleen, kidneys, lungs, heart, testis and brain. They didn't find any specific disorder in liver, kidney and blood factors during one month and also no weight changes were observed [14]. Hafeli and Pauer intraspinally injected 0.5 mg of magnetic microspheres coated with polylactic acid into rats. They didn't realize any toxic effect or abnormality in animals' behaviour. Rats' growth was reported normal and no mortality was observed during one-year post-injection [11].

On the other hand, Some researchers reported that intravenous injection of 100 µl of DMSA coated magnetic iron oxide nanoparticles (9 nm) into mice, while crossing the blood-air dam and entering lungs (respiratory bronchioles and alveolar sac) led to an inflammatory response, but the severity of these changes reduced after three months [10,13]. In another study, 50 µl of poly-aspartic acid-coated iron oxide nanoparticles were intravenously injected into mice which caused inflammatory responses and also lymphocytes, monocytes and neutrophils increase. In addition, some disorders were occurred in the maturation process of red blood cells due to the presence of this substance in red bone marrow and led to cytotoxic and genotoxic effects. However, such effects occur from first to fifteenth-day post-injection and then reside until one month [8]. Bourrinet et al observed some effects such as dark spots on rats' body, a breathing disorder, red spots around the nose, fetal skeletal abnormalities and weight loss after the intravenous injection of dextran-coated Fe_3O_4 nanoparticles. They also reported ataxia, decreased activity, exophthalmia, emesis, salivation, lacrimation, mucus colourless stool and yellow eyes after single intravenous injection of 20 and 200 mg Fe/kg concentrations into dogs [15]. In another study, a single intravenously injection of 100 µl of DMSA coated magnetic liquid into mice led to rapid death due to the accumulation of high amount of nanoparticles in lungs and damage in pulmonary capillaries (respiratory problems) [16].

The most important and remarkable point which is mentioned in most studies is small amount usage of iron oxide nanoparticles in medicine has not any severe effect and should be reversible in most cases.

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