# The Effect of Bovine Serum Albumin and Dimercaptosuccinic Acid Coated Iron Oxide Nanoparticles on Liver and Renal Function in Mice

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## *ABSTRACT*

***Objective:*** *To evaluate the effect of iron oxide ($Fe_3O_4$) nanoparticles coating by biocompatible compound on their toxicity and also comparison with uncoated nanoparticles.*
***Methods:*** *The co-precipitation method was used in order to synthesize $Fe_3O_4$ nanoparticles. The synthesized nanoparticles were coated by bovine serum albumin (BSA) and dimercaptosuccinic acid (DMSA) and the coating interactions were investigated by fourier transform infrared spectroscopy (FTIR). Nanoparticles properties were evaluated by alternating gradient force magnetometer (AGFM), transmission electron microscope (TEM) and X-ray diffraction (XRD). Toxicity assessment of nanoparticles were studied in mice by intraperitoneal injections. Liver enzymes (SGPT, SGOT, ALP and LDH) and also renal kidney factors (uric acid, creatinine and urea) were measured 7, 15 and 30 days post-injection.*
***Results:*** *The nanoparticles size was around 5 to 11 nm. Results showed that the amount of urea and creatinine were significantly increased seven days post-injection in the group receiving high doses. The amount of uric acid reduced significantly in all groups in comparison with the control. Iron oxide, nanoparticles also caused histologic changes in the kidney including accumulation in the glomerulus, nephron wall cells, internal nephron canal and kidney blood vessels and also inflammatory cells in glomerular capillaries and degeneration of proximal and distal tubules. Some liver enzymes were changed due to the injection of both uncoated and coated nanoparticles to mice. The liver enzymes changes were more considerable in the groups receiving DMSA or DMSA coated in comparison with the groups receiving BSA or BSA coated. There is no irreversible effect in concentrations less than 200 mg/kg for all control and treated groups.*
***Conclusion:*** *It seems that $Fe_3O_4$ nanoparticles have a short-term effect on kidney and liver function. By the gradual elimination of particles uptake into the kidney, most effects disappeared during a month.*


**Keywords:** Bovine serum albumin, dimercaptosuccinic acid, iron oxide nanoparticle, liver enzyme, renal function

# Efecto de la Albúmina de Suero Bovino y las Nanopartículas de Óxido de Hierro Revestidas con Ácido Dimercaptosuccínico Sobre el Hígado y la Función Renal en Ratones

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## *RESUMEN*

***Objetivo:*** *Evaluar el efecto del revestimiento de las nanopartículas de óxido de hierro (Fe3O4) con un compuesto biocompatible sobre su toxicidad, y su comparación con nanopartículas sin recubrimiento.*
***Métodos:*** *Se utilizó el método de co-precipitación para sintetizar las nanopartículas de Fe3O4. El nanopartículas sintetizadas fueron revestidas con albúmina de suero bovino (ASB) y ácido dimer-captosuccínico (ADMS), y las interacciones del revestimiento fueron investigadas mediante por espectrometría infrarroja de transformación de Fourier (FTIR). Las propiedades de las nanopartículas fueron evaluadas mediante el magnetómetro de gradiente de campo alterno (AGFM), el microscopio electrónico de transmisión (TEM), y la difracción de rayos X (DRX). La evaluación de la toxicidad de las nanopartículas fue estudiada en ratones por medio de inyecciones intraperitoneales. Las enzimas hepáticas (SGPT, SGOT, ALP, y LDH) y también los factores renales (ácido úrico, creatinina y urea) fueron medidos 7, 15 y 30 días después de la inyección.*


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***Resultados:*** *El tamaño de las nanopartículas fue de alrededor de 5 a 11 nm. Los resultados mostraron que la cantidad de urea y creatinina fueron significativamente mayores siete días después de la inyección en el grupo que recibió dosis altas. La cantidad de ácido úrico se redujo significativamente en todos los grupos en comparación con el control. Las nanopartículas de óxido de hierro, también causaron cambios histológicos en el riñón, incluyendo acumulación en el glomérulo, las células de la nefrona, el canal interno de la nefrona, y los vasos sanguíneos del riñón, así como las células inflamatorias en los capilares glomerulares y la degeneración de los túbulos proximales y distales. Algunas enzimas del hígado fueron cambiadas, debido a la inyección de nanopartículas sin revestir y revestidas en los ratones. Los cambios de las enzimas hepáticas eran más considerables en los grupos que recibieron ADMS o ADMS revestido, en comparación con los grupos que recibieron ASB ó ASB revestido. No hay ningún efecto irreversible en concentraciones menores de 200 mg/kg para ninguno de los grupos tratados o de control.*
***Conclusión:*** *Todo parece indicar que las nanopartículas Fe3O4 tienen un efecto a corto plazo sobre la función renal y hepática. Con la eliminación gradual de la absorción de las partículas en el riñón, la mayor parte de los efectos desaparecieron en un mes.*

**Palabras claves:** Albúmina de suero bovino, ácido dimercaptosuccínico, nanopartículas de óxido de hierro, enzimas hepáticas, función renal

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## INTRODUCTION

A high proportion of surface to volume of the nanoparticles in comparison of bulk samples, have made them adequate to be used in many medical and industrial applications. There are considerable studies on vast application of $Fe_3O_4$ nanoparticles such as in cancer therapy, magnetic resonance (MR) imaging tumour therapy, drug delivery (1−3). There are many methods that can be used for synthesizing nanoparticles such as co-precipitation, low temperature, solid state reaction and microwave method (4−7).

The ultra small size of nanoparticles provided for fast distribution in many organs and considerable cellular uptake phenomenon (8). Nowadays, nanoparticles are coated with biocompatible materials in order to increase their stability in blood circulation and biological systems. The presence of these coatings on the surface of nanomaterials increases the cell entrance and reduces their toxicity effects. In order to coat nanoparticles, some biocompatible materials were used such as bovine serum albumin (BSA), dimercaptosuccinic acid (DMSA) $C_4S_2O_4H_6$, dextran, polyethylene glycol, chitosan and aspartic acid (9−12).

There are small numbers of studies on $Fe_3O_4$ toxicity especially under *in vivo* conditions and also, some studies reported controversial results. For instance, some researchers have reported non-toxicity under *in vivo* conditions and some others have shown minimal toxicity at same concentrations (13−16).

The kidney is an important organ for excretion of waste material from the body. Moreover, electrolyte and water balance are regulated *via* this organ. The estimation of its histology and some waste metabolic products excreted *via* the kidneys provide useful information about the health of this organ. The urea and creatinine are waste products of protein metabolism that are excreted through the kidney and increase of these factors is a sign of kidney damage. The level of blood creatinine is proportional to the glomerular filtration rate. Moreover, urea plays an important role in the metabolism of compounds containing protein in animals' bodies. Uric acid is the metabolic end product of purine metabolism in humans and has antioxidant properties but can be a pro-oxidant. It is also a weak organic acid that increases in many diseases.

Dimercaptosuccinic acid is a nontoxic substance recently used in some patients in order to absorb additional elements of the body [chelating agent] (14). Bovine serum albumin is a serum albumin protein derived from cows. It is often used as a protein concentration standard. Using these substances creates an anionic coating around the nanoparticle surface and therefore decreases direct contact with cells and cellular components which led to change in the toxicity effects. Moreover, they increase tissue distribution and cell absorption (16).

## MATERIALS AND METHODS

### Iron oxide nanoparticles synthesis

Iron oxide nanoparticles were produced by a co-precipitation method. For this purpose, the solutions of $FeCl_3$ (0.02 M equals to 5.41 g), $FeCl_2$ (0.01 M equals to 1.98 g) and NaOH (0.08 M equals to 3.2 g) [all from Merck company] were prepared in distilled deionized water. In this method, $FeCl_2$ solution was poured into a triple neck round balloon and meanwhile, $FeCl_3$ solution was added to the same balloon under vigorous magnetic stirring. Then, every three or four seconds, one droplet of ME solution was added *via* magnetic stirrer. Finally, NaOH solution was added to the balloon in the same way (17). The resulting solution was washed by deionized water and then was centrifuged in order to remove any aggregate as impurity. All processes were done at room temperature.

**Nanoparticles coating by bovine serum albumin and dimercaptosuccinic acid**

0.5 g of BSA was diluted to 50 mL normal saline and the solution was added to 100 mL of $Fe_3O_4$ solution which was prepared before and allowed the interaction to be completed for three hours under rapid stirring with ultrasonic. The coated nanoparticles were separated from the uncoated nanoparticles and extra BSA by centrifugation for 30 minutes. Then, for more accuracy, 100 mL of distilled water was added and the centrifugation was repeated. Dimercaptosuccinic acid coated Fe3O4 nanoparticles were synthesized by similar process but with less ultrasonic time. All processes were done at room temperature. It mean that the same processes were done except for, ultrasonic process which was done just for one-hour (17−19).

**Assessment of synthesized samples properties**

Transmission electron microscope (TEM) was used for size and size distribution. X-ray Diffraction (XRD), Bruker D8 ADVANCE $\lambda = 0.154$ nm Cu Kα radiation, was used in order to evaluate size and crystalline structure. The crystalline size was calculated from Debye-Scherrer equation. Alternating gradient-force magnetometer (AGFM), Meghnatis Daghigh Kavir Co, Iran was used to evaluate the magnetic properties of the nanoparticles. The coating chemical interactions were assessed by fourier transform infrared spectroscopy (FTIR), JASCO FT/IR-680 PLUS (20).

**Breeding animals and treatments**

240 Balb/c mice were prepared from RAZI Vaccine and Serum Research Institute. They were maintained under controlled temperature, 12 hours light and 12 hours dark conditions for one week before beginning the experiments for adaption to laboratory conditions. All procedures in this study were in accordance with the institutional animal care. Mice were divided into 16 equal groups (each group contains 15 mice). One group was injected with normal saline as a control group and the 15 remaining groups received $Fe_3O_4$, DMSA, BSA, $Fe_3O_4$ @ DMSA and $Fe_3O_4$@BSA. Different concentrations of 50, 100 and 200 mg per kg of mice weight were intra-peritoneally injected.

**Renal and liver factors measurement**

Animals were anaesthetized at the mentioned times and blood samples were collected directly from the animal's heart. Blood samples were poured into the special pipes which contain edetic acid (EDTA) anti coagulation agent. The serum biochemical parameters such as urea, uric acid, creatinine, serum glutamic pyruvate transaminase (SGPT), serum glutamic oxaloacetic transaminase (SGOT), lactate dehydrogenase (LDH) and alkaline phosphatase (ALP) were measured by Automatic Analyser (RA1000 Technicon, America).

**RESULTS**

**Physical properties of coated iron oxide nanoparticles**

Figure 1 indicates the XRD pattern of the DMSA coated and BSA coated $Fe_3O_4$ nanoparticles. As can be seen, both of the samples are single phase and also have the ferrite spinel structure. The intensity of XRD background toward peak is higher in the sample coated with BSA in comparison with that of DMSA. It is probably due to the DMSA and BSA structures. The mean size of the particles was determined by Debye-Scherer formula. It was calculated as 17 nm for DMSA coated and 25 nm for BSA coated $Fe_3O_4$ nanoparticles.

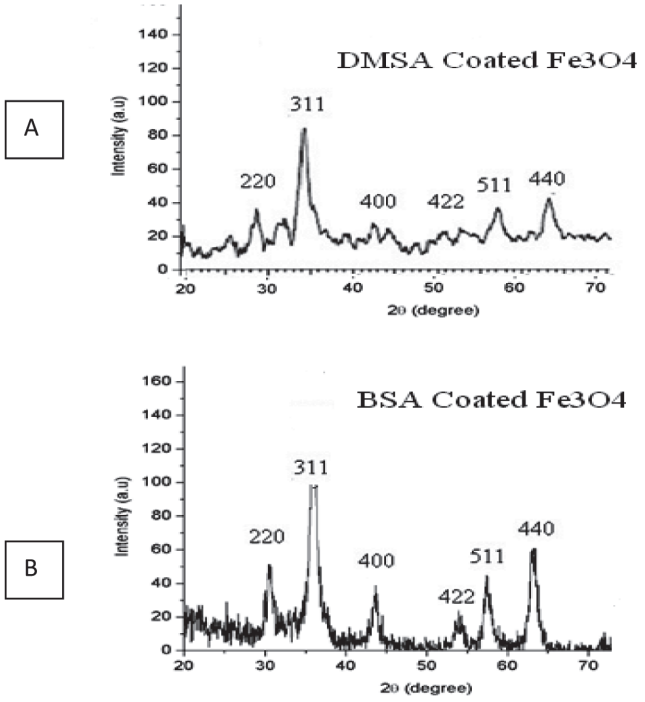


Fig. 1: X-ray diffraction pattern: (a) dimercaptosuccinic acid coated $Fe_3O_4$, (b) bovine serum albumin coated $Fe_3O_4$

Transmission electron microscope photograph of the uncoated $Fe_3O_4$ nanoparticles is shown in (Fig 2). This photograph indicates that the sizes of the particles are around 10 nm with approximately uniform size distribution. This is compatible with the results of the XRD patterns (Fig. 1) because the particle size increases by the coating process. Magnetic properties of the nanoparticles were investigated by AGFM and it was proven that both samples were more super-paramagnetic. The saturation magnetization was determined by extrapolation of the magnetization curve (when $1/H \rightarrow 0$). It measured 27 and 23 emu/gr for DMSA and BSA coated nanoparticles respectively [AGFM curves are not shown here] (20).

Fourier transform infrared spectroscopy curves of the $Fe_3O_4$, DMSA coated and BSA coated $Fe_3O_4$ nanoparticles are demonstrated in (Fig. 3).

It can be seen that, 1628 and 3419 peaks in the $Fe_3O_4$ curve, are related to OH junctions and it can be concluded that there is water molecule in the material structure. The 581 peak indicates that the spinel structure was formed and will be seen as compatible with the XRD curve results. Moreover, 1619 and 1376 peaks in DMSA coated $Fe_3O_4$ nanoparticles curve,

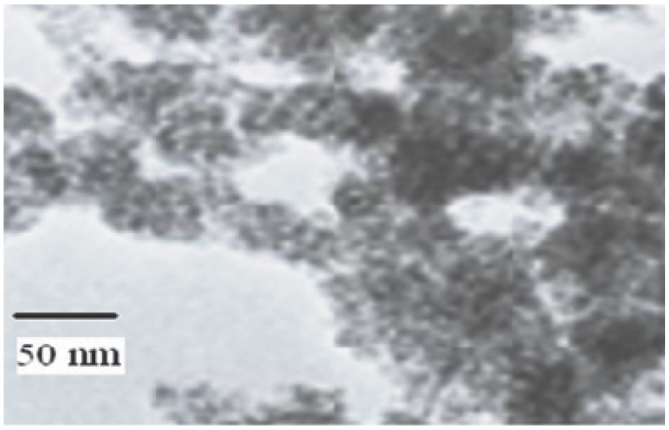


Fig. 2: Transmission electron microscope photograph of the $Fe_3O_4$ nanoparticles.

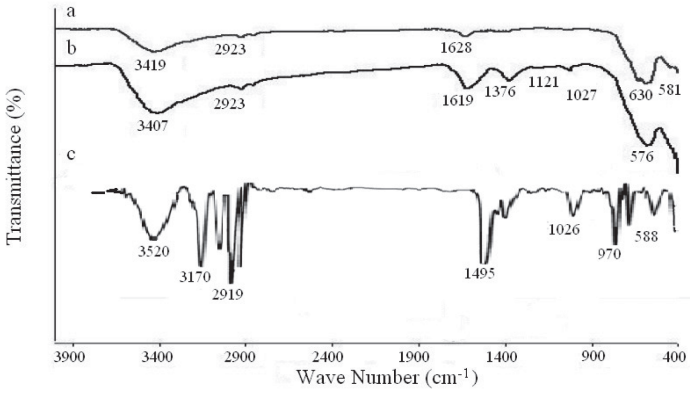


Fig. 3: (a) FTIR curve of $Fe_3O_4$ (b) FTIR curve of $Fe_3O_4$@DMSA (c) FTIR curve of $Fe_3O_4$@BSA

FTIR: Fourier transform infrared spectroscopy

are related to the asymmetry and symmetry stresses and stretches of carbonyl. By considering that these peaks are in a close relation with the 1699 and 1421 peaks in the DMSA curve, it is proven that the DMSA has coated the surface of the $Fe_3O_4$ nanoparticles. Furthermore, the 581 peak decrease is another reason for this conjunction (20). By the same way, it can be concluded that BSA has also coated the surface of the $Fe_3O_4$ nanoparticles. For instance, 2919 and 3520 peaks in the BSA coated $Fe_3O_4$ nanoparticles curve are the deformed peaks of 2923 and 3419 in $Fe_3O_4$ curve.

**Renal factors measurement**

Table 1 shows the results of renal factors measurement for 200 mg/kg concentration. It can be seen that the amounts of urea and creatinine increased significantly seven days post-injection in the group receiving 200 mg/kg. Moreover, the amount of uric acid decreased significantly eight days post-injection in all treated groups in comparison with control and placebo groups. On the other hand, the results of 15 and 30 days post-injection showed that these values returned to normal.

Table 1: Renal factors measurement seven days post-injection (200 mg/kg concentration)

| | Urea (mg/dL) | Uric Acid (mg/dL) | Creatinine (mg/dL) |
|---|---|---|---|
| Control | 42.25 ± 3.10 | 2.22 ± 0.18 | 0.52 ± 0.13 |
| BSA | 41.45 ± 2.20 | 2.15 ± 0.25 | 0.58 ± 0.15 |
| DMSA | 43.5 ± 2.32 | 2.42 ± 0.13 | 0.60 ± 0.13 |
| $Fe_3O_4$ | 40.62 ± 3.62 | $^{\alpha}$1.03 ± 0.30 | 0.45 ± 0.05 |
| $Fe_3O_4$@BSA | 41.05 ± 4.17 | $^{\alpha}$1.20 ± 0.22 | 0.42 ± 0.09 |
| $Fe_3O_4$@DMSA | $^{*}$52.11 ± 7.40 | $^{\alpha}$1.23 ± 0.26 | $^{*}$0.63 ± 0.17 |

α:Significant reduction of uric acid level ($p < 0.001$).
*:Significant increase of urea and creatinine levels ($p < 0.001$)

BSA: bovine serum albumin; DMSA: dimercaptosuccinic acid; $Fe_3O_4$: iron oxide; $Fe_3O_4$@BSA: iron oxide-bovine serum albumin; $Fe_3O_4$@DMSA: iron oxide-dimercaptosuccinic acid

Table 2: Renal factors measurement 15 days post-injection (200 mg/kg concentration)

| | Urea (mg/dL) | Uric Acid (mg/dL) | Creatinine (mg/dl) |
|---|---|---|---|
| Control | 41.45 ± 2.20 | 2.15 ± 0.25 | 0.58 ± 0.15 |
| BSA | 41.55 ± 2.22 | 2.17 ± 0.27 | 0.61 ± 0.18 |
| DMSA | 42.5 ± 2.21 | 2.52 ± 0.12 | 0.61 ± 0.14 |
| $Fe_3O_4$ | 41.74 ± 2.45 | 1.82 ± 0.75 | 0.49 ± 0.09 |
| $Fe_3O_4$@BSA | 43.40 ± 3.28 | 1.76 ± 0.15 | 0.43 ± 0.07 |
| $Fe_3O_4$@DMSA | 47.17 ± 4.66 | 1.85 ± 0.37 | 0.57 ± 0.13 |

Table 3: Renal factors measurement 30 days post-injection (200 mg/kg concentration)

| | Urea (mg/dL) | Uric Acid (mg/dL) | Creatinine (mg/dl) |
|---|---|---|---|
| Control | 41.35 ± 2.27 | 2.18 ± 0.29 | 0.51 ± 0.17 |
| BSA | 41.52 ± 2.25 | 2.27 ± 0.20 | 0.63 ± 0.21 |
| DMSA | 41.7 ± 2.20 | 2.42 ± 0.15 | 0.63 ± 0.10 |
| $Fe_3O_4$ | 43.63 ± 3.95 | 2.24 ± 0.27 | 0.46 ± 0.08 |
| $Fe_3O_4$@BSA | 46.88 ± 6.02 | 2.34 ± 0.16 | 0.52 ± 0.08 |
| $Fe_3O_4$@DMSA | 44.24 ± 1.67 | 2.21 ± 0.17 | 0.52 ± 0.11 |

Figure 4 shows accumulated iron oxide nanoparticles in the stained kidney tissue sections seven days post-injection. As can be seen, $Fe_3O_4$ nanoparticles were found in the glomerulus, nephron wall cells, internal nephron canal and kidney blood vessels. These results indicate that, the iron oxide nanoparticles were passed through the membrane of different cells.

The accumulation of neutrophils and eosinophils in glomerular capillaries (due to the capillary infiltration), the degeneration in proximal and distal tubule walls and the accumulation of dense eosinophilic material in proximal and distal tubules (due to the congestion of eosinophils in these tubules) were observed seven days post-injection. These disorders were not observed 30 days post-injection (the results are not shown here).

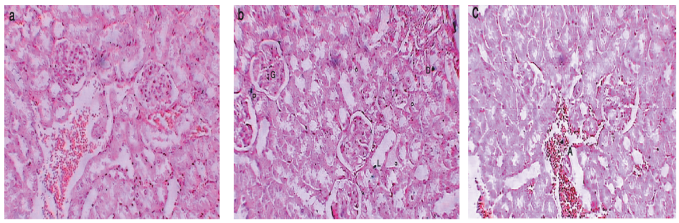

Fig. 4: The kidney tissue sections (Perl's Method) seven days post-injection. (Magnification 40 x)
(a) Control group. (b, c) 100 mg/kg treated group. Dark blue dots show the accumulation of iron oxide nanoparticles in the glomeruls (G), nephron wall cells (P & D), internal nephron canal (L) and kidney blood vessels (V).

### Liver enzymes measurement

Serum glutamic oxaloacetic transaminase, SGPT, ALP and LDH liver enzymes were measured 7, 15, 30 days post-injection in all 16 groups. The results on days 7 and 15 are similar and therefore the results of day 7 are not shown here. Figures 5−8 show the SGOT, SGPT, ALP and LDH measurement results 15 days post-injection.

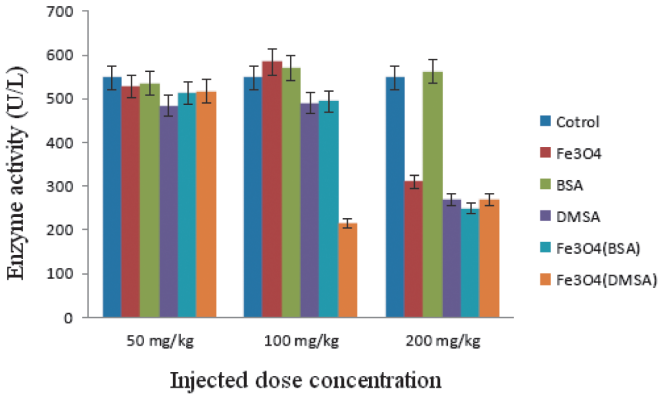


Fig. 5: Serum glutamic oxaloacetic transaminase measurement results 15 days post-injection.

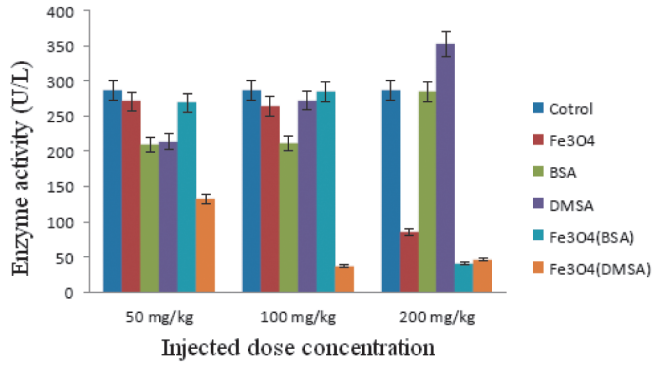


Fig. 6: Serum glutamic pyruvate transaminase measurement results 15 days post-injection.

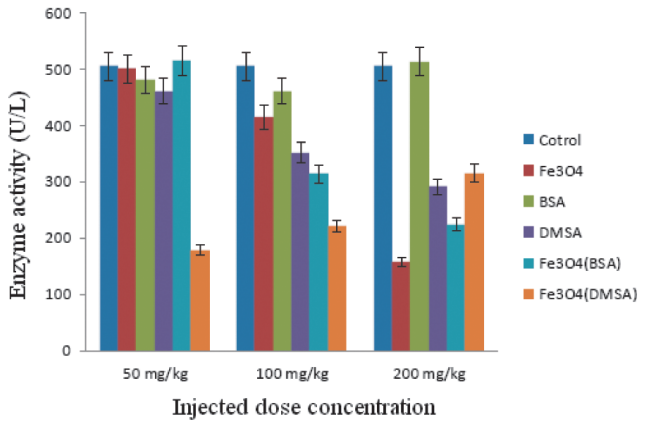


Fig. 7: Alkaline phosphatase measurement results 15 days post-injection.

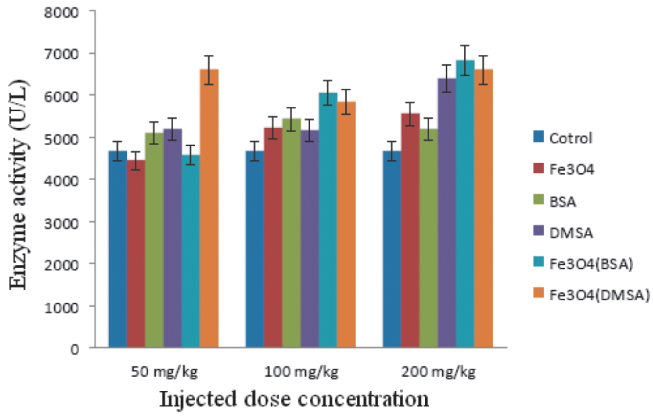


Fig. 8: Lactate dehydrogenase measurement results 15 days post-injection.

As can be seen, there is no meaningful change in the groups receiving less than 100 mg/kg BSA, DMSA and uncoated $Fe_3O_4$ nanoparticles in comparison with control. There is significant change for the DMSA coated nanoparticles treated group even in 50 mg/kg concentration but BSA coated nanoparticles treated group shows meaningful change just in 200 mg/kg concentration. Therefore, it can be said that BSA has high compatibility with biological systems. Also, there is no significant change in mice's weight in all the groups during a month.

## DISCUSSION

The findings seem to prove that coated nanoparticles affect liver function more in comparison with uncoated iron oxide nanoparticles. This probably occurred, due to better stability in blood circulation and consequently more penetration in different organs and cells.

By surveying the results of 30 days post-injection, it can be concluded that most values are returning to the normal value

and it is expected that all measured enzymes return to normal value in the near future (results are not shown here). This point shows that coated and uncoated iron oxide nanoparticles do not create any irreversible effect or disorder in liver function even in high doses (200 mg/kg).

These results are similar to that of Kim *et al*, who injected less than 100 mg/kg concentrations of silica ($SiO_2$) coated nanoparticles ($CoFe_2O_4$) intra-peritoneally in mice and determined the presence and distribution of nanoparticles in mice organs. No specific disorder was found in liver enzymes, 30 days post-injection and also no weight changes were observed (16). Hafeli and Pauer injected poly lactic acid coated $Fe_3O_4$into rats intra-spinally. They did not observe any mortality, toxicity or abnormality in animals' behaviour during one year post-injection. Animal growth and weight were reported normal (15).

On the other hand, Sadeghiani *et al* reported inflammatory responses due to the intravenous injection of poly aspartic acid coated $Fe_3O_4$ nanoparticles into mice. Moreover, they observed that lymphocytes, monocytes and neutrophils increased and also some disorders in the maturation process of red blood cells occurred. These effects occurred during the first to 15$^{th}$ day post-injection and remained for one-month (13). In another study, DMSA coated Fe3O4 nanoparticles were intravenously injected into mice. The nanoparticles were interred into lungs (respiratory bronchioles and alveolar sac) and cause inflammatory responses but the severity of these changes reduced after three months (14). The most important and remarkable point mentioned in most studies is that small amounts of $Fe_3O_4$ nanoparticles in medicine do not create any severe effect.

The results of this study indicate that, most of liver enzymes were actively changed significantly, but, this was temporary and most returned to their normal range after a month. From the findings here, low concentrations *in vivo* of $Fe_3O_4$ nanoparticles (less than 200 mg/kg) do not create any serious or severe toxic effect.

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