

## Evaluation of Iron Oxide Nanoparticles Toxicity on Liver Cells of BALB/c Rats

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### Abstract

**Background:** Because of their unique magnetic properties, Fe<sub>3</sub>O<sub>4</sub> nanoparticles (Fe<sub>3</sub>O<sub>4</sub>-NPs) have extensive applications in various biomedical aspects. Investigation of the possible adverse aspects of these particles has lagged far behind their fast growing application.

**Objectives:** The current study aimed to evaluate the toxicity of Fe<sub>3</sub>O<sub>4</sub>-NPs in the liver of mice.

**Materials and Methods:** In the present clinical trial, 90 BALB/c mice were randomly divided in 15 groups. Five control groups were fed by usual water and food. Five placebo groups were gavaged with physiological serum in doses of 25, 50, 75, 150, and 300 micrograms per gram of body weight (µg/gr). Five experimental groups were gavaged with Fe<sub>3</sub>O<sub>4</sub>-NPs, in doses of 25, 50, 75, 150, and 300 µg/gr. This pattern was repeated every other day, for 3 days. Then, the levels of liver enzymes [alanine transaminase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP)] were compared between these groups. The histological alterations of livers were examined, as well. For statistical analysis, Kruskal-Wallis and Mann-Whitney, with type I Bonferroni correction, as post-hoc, have been used.

**Results:** The administration of 150 and 300 µg/gr doses of Fe<sub>3</sub>O<sub>4</sub>-NPs were associated with significant elevation in liver enzymes, compared to controls (P < 0.0001). Furthermore, the histopathological effects were observed in the liver tissue of these groups. However, in groups treated with lower doses of Fe<sub>3</sub>O<sub>4</sub>-NPs, no significant adverse effect was observed.

**Conclusions:** Based on our results, the administration of Fe<sub>3</sub>O<sub>4</sub>-NPs causes dose dependent adverse effects on liver.

**Keywords:** Ferric Oxide, Nanoparticles, Aspartate Aminotransferases, Alanine Transaminases, Alkaline Phosphatase, Hepatocytes, Liver

### 1. Background

Nanoparticles (NPs) have very specific chemical and physical characteristics in relation to their size, shape and high proportion of surface to volume. These characteristics have made them appropriate for use in multiple medical and biological applications, such as contrast media for magnetic resonance imaging (MRI) (1), immunoassays (2), hyperthermia (3), tissue repair (4), cellular therapy (4) and drug delivery (5). Nanoparticles, consisting of elements such as iron, nickel and cobalt, which exhibit magnetic properties, are called "magnetic NPs". Magnetic NPs offer several attractive possibilities, as they can be internally manipulated under the influence of an external magnetic field. The NPs have been shown to be taken directly into the bloodstream, following inhalation and ingestion exposure. Typically, magnetic NPs distribute to the liver (80-90%), spleen (5-8%) and bone marrow (1-2%) (6). As the liver is the most important detoxification organ, it is reached by the highest level of NPs concentration, over all the other tissues (7, 8). Therefore, the liver

can be seen as a target organ to identify adverse effects of in vivo exposure to iron oxide NPs (IONPs).

Alanine transaminase (ALT) and aspartate aminotransferase (AST) are two of the most reliable markers of hepatocellular injury or necrosis. Their levels can be elevated in a variety of hepatic disorders. Of the two, ALT is thought to be more specific for hepatic injury, because it is present mainly in the cytosol of the liver and, in low concentrations, elsewhere. The AST has cytosolic and mitochondrial forms and is present in tissues of the liver, heart, skeletal muscle, kidneys, brain, pancreas, lungs and in white and red blood cells. Alkaline phosphatase (ALP), which is present in a number of tissues, including liver, bone, intestine and placenta, is measured to detect the liver disease or bone disorders. It is known that, following their exposure, NPs are primarily readily taken up by hepatocytes and Kupffer cells (specialized macrophages located in the liver) (9). Because of their unique properties, several of these NPs, such as IONPs, have been approved for clinical use.

The application of IONPs has been practiced for nearly 40 years (10) and, in the last decade, they have received enormous attention in various fields of biotechnology and life sciences. The two promising forms of IONPs are magnetite ( $\text{Fe}_3\text{O}_4$ ) and its oxidized form, maghemite ( $\gamma\text{-Fe}_3\text{O}_4$ ), since their biocompatibility has already been proven (11).

## 2. Objectives

Although these nanoparticles are currently being widely used in modern technology, there is a serious lack of information concerning their impact on human health and environmental implications. Concerning the central role of liver in the clearance of NPs, we aimed to investigate the effect of  $\text{Fe}_3\text{O}_4$ -NPs on this organ, by measuring the changes in level of liver enzymes, as well as histopathological alteration of liver tissue (including hepatocytes and Kupffer cells) in mature male mice (BALB/c race).

## 3. Materials and Methods

### 3.1. Preparation and Characterization of Magnetite Nanoparticles

The current clinical trial study was performed in Islamic Azad University, Department of Biology, Science and Research Branch, Iran, from March 2014 to September 2014. The  $\text{Fe}_3\text{O}_4$ -NPs (diameter 20-30 nm) were purchased from the company of Nano Pasargad Novin (Iran, Tehran). Twenty five grams of these particles were suspended in 10 mL of PBS (pH = 7.5) to create a stock solution and, at the time of experiment, were diluted to required doses. Ultra sonication was performed to disperse the coagulated particles, just before each administration, therefore excluding any disturbance of the heterogeneity of nanoparticles.

### 3.2. Experimental Animals

Ninety adult male mice of BALB/c strain, weighing  $23 \pm 2$  grams were purchased from the Razi Vaccine and Serum Research Institute (Karaj, Iran). The laboratory animal facility was maintained under a 12 hours light/dark cycle, at a temperature of  $22 \pm 2^\circ\text{C}$ , and a relative humidity of 20-50%. All methods performed in this study were in accordance with regulatory guidance on the care and use of experimental animals. Experimental animals were randomly divided into 15 groups, as follows: five control groups (comprising six mice in each group) fed by usual water and food. Five experimental groups (comprising six mice in each) were gavaged with  $\text{Fe}_3\text{O}_4$ -NPs, in doses of 25, 50, 75, 150 and 300  $\mu\text{g}/\text{gr}$ . Five control groups that referred to placebo (comprising six mice in each) were gavaged with physiological serum, in doses of 25, 50, 75, 150 and 300  $\mu\text{g}/\text{gr}$ . This pattern was repeated every other day, for 3 days.

### 3.3. Serum Biochemical Analysis

One day after 3 days of treatment with  $\text{Fe}_3\text{O}_4$ -NPs, the mice were anesthetized with ether. For biochemical anal-

ysis, the heart blood was collected and the serum was isolated by centrifugation (3000 rpm for 20 minutes). Then, concentrations of liver enzymes, including ALT, AST and ALP were determined using marketed reagent kits (Pars Azmoon, Tehran, Iran) and autoanalyzer system (Hitachi Ltd., Tokyo, Japan).

### 3.4. Histological Examinations

All mice fed with  $\text{Fe}_3\text{O}_4$ -NPs were then sacrificed by giving deep ether anesthesia and were operated to remove their liver. The tissue samples were fixed in buffered neutral 10% formaldehyde, decalcified in 5% nitric acid for 7 days, dehydrated stepwise through ascending series of alcohol solutions and, finally, degreased in xylene. The tissues were then embed in paraffin block, sliced at 5  $\mu\text{m}$ , by microtome and stained using hematoxylin and eosin (HE) stains. Cells were observed under a CH-30 optical microscope (Olympus, Tokyo, Japan) and histological images were captured and analyzed. Then, the hepatocytes and Kupffer cells were counted per area.

### 3.5. Statistical Analysis

The data were analyzed using Kruskal-Wallis and Mann-Whitney with type I Bonferroni correction as post hoc. Continuous variables are presented as the mean  $\pm$  SD and Median. The confidence level of current study is 95%. A  $P < 0.0001$  was considered as statistically significant.

## 4. Results

### 4.1. Hepatic Histological Alterations

Hepatic tissue pieces that stained by HE were studied under light microscope, with magnification of 400 $\times$ . Spotty necrosis of hepatocytes, with infiltration of lymphocytes and neutrophils in the area and a plethora of Kupffer cells were observed in mice treated with 150 and 300  $\mu\text{g}/\text{gr}$  doses of  $\text{Fe}_3\text{O}_4$ -NPs (Figure 1). Furthermore, histopathological examination indicated the central venous congestion and hypertrophied hepatocytes in the livers of these two groups (Figure 2). Five percent of hepatocytes of mice treated with these doses showed regenerative activities. Three days administration of  $\text{Fe}_3\text{O}_4$ -NPs in doses of 150 and 300  $\mu\text{g}/\text{gr}$  were also associated with a significant increase in the numbers of Kupffer cells ( $P < 0.0001$ ), compared to unexposed controls. However, no significant increase in the number of these cells was determined in mice treated with 25, 50 and 75  $\mu\text{g}/\text{gr}$  of  $\text{Fe}_3\text{O}_4$ -NPs (Table 1).

In comparison with unexposed controls, consumption of  $\text{Fe}_3\text{O}_4$ -NPs in both 150 and 300  $\mu\text{g}/\text{gr}$  doses lead to significant decrease in the number of hepatocytes compared with unexposed controls ( $P < 0.0001$ ). However, no significant changes were observed in the number of hepatocytes of mice treated with 25 and 50  $\mu\text{g}/\text{gr}$   $\text{Fe}_3\text{O}_4$ -NPs (Table 1).

The number of venous cavities and the width of hepatic vein showed a significant increase in mice treated with

Fe<sub>3</sub>O<sub>4</sub>-NPs in 150 and 300 µg/gr doses, compared to untreated groups (Table 1).

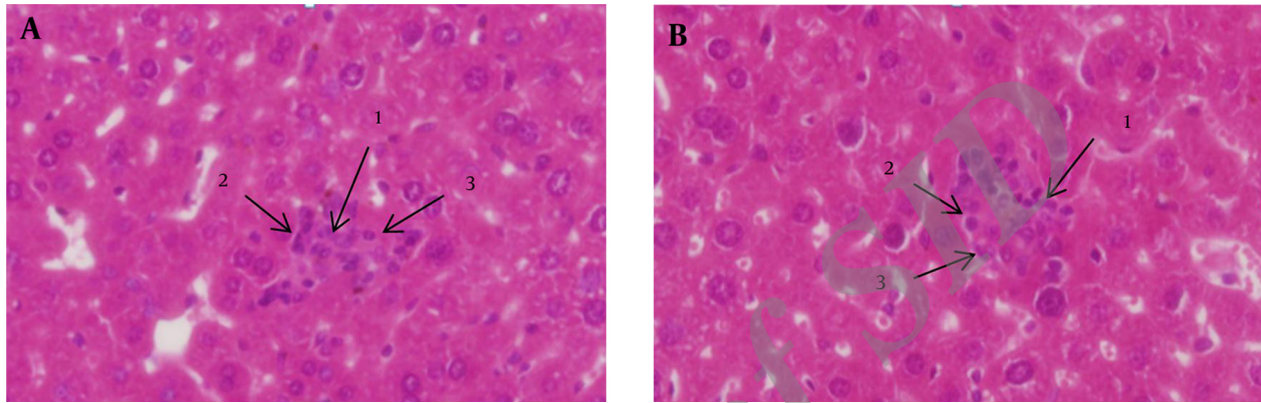
#### 4.2. Hepatic Enzymes Alterations

The serum level of ALT in the groups treated with 150 and 300 µg/gr doses of Fe<sub>3</sub>O<sub>4</sub>-NPs were shown to be significantly higher, compared to unexposed controls (P < 0.0001). Administration of Fe<sub>3</sub>O<sub>4</sub>-NPs in three other concentrations

(25, 50 and 75 µg/mg) had not significant effect on ALT level, in comparison with unexposed controls (Table 2).

In addition, the levels of both AST and ALP enzymes showed a significant increase in mice treated with 150 and 300 µg/gr Fe<sub>3</sub>O<sub>4</sub>-NPs, compared to unexposed controls (P < 0.0001). However, no significant difference was observed in the level of these enzymes, when comparing the groups of mice treated with 25, 50 and 75 µg/mg doses of these NPs with the control groups (Table 2).

**Figure 1.** Histopathology of Liver in Mice Treated With 300 µg/gr Doses of Magnetite Nanoparticles



A, Histopathology of liver in mice treated with 300 µg/gr doses of Fe<sub>3</sub>O<sub>4</sub>-NPs; B, 1) Spotty necrosis, 2) Neutrophil and 3) Lymphocyte; Abbreviations: Fe<sub>3</sub>O<sub>4</sub>-NPs, Magnetite Nanoparticles.

**Table 1.** Data Analysis Using Kruskal-Wallis Method<sup>a</sup>

Groups	Intact Control	Placebo Control	25 µg/gr dose	50 µg/gr dose	75 µg/gr dose	150 µg/gr dose <sup>b</sup>	300 µg/gr dose <sup>b</sup>	P value
<b>Venous Cavities, n</b>	58.2500 ± 1.40955	58.0000 ± 1.37649	58.2500 ± 0.85070	59.0000 ± 0.91766	59.2500 ± 1.06992	43.1000 ± 1.88903	37.350 ± 1.18210	< 0.0001
<b>Hepatic Vein Width, µm</b>	11.9500 ± 1.09904	12.2000 ± 0.83351	12.3500 ± 0.98809	13.0500 ± 1.09904	13.1500 ± 1.18210	18.2500 ± 1.06992	26.4500 ± 0.82558	< 0.0001
<b>hepatocytes, n</b>	316.85 ± 18.49687	308.95 ± 14.92605	317.4500 ± 14.89428	324.8000 ± 14.48629	331.5000 ± 9.52283	234.1500 ± 3.43779	211.2500 ± 2.17340	< 0.0001
<b>Kupffer cells, n</b>	31.5000 ± 1.84961	30.7500 ± 1.55174	31.5000 ± 1.53897	32.3500 ± 1.46089	33.5000 ± 1.14708	61.9000 ± 4.15363	71.050 ± 2.06410	< 0.0001

<sup>a</sup>Evaluating the effect of different magnetite nanoparticles (Fe<sub>3</sub>O<sub>4</sub>-NPs) doses on various histological factors of liver indicated that administration of Fe<sub>3</sub>O<sub>4</sub>-NPs in 150 and 300 µg/gr doses was associated with significant histopathological changes.

<sup>b</sup>Significant difference (P < 0.0001).

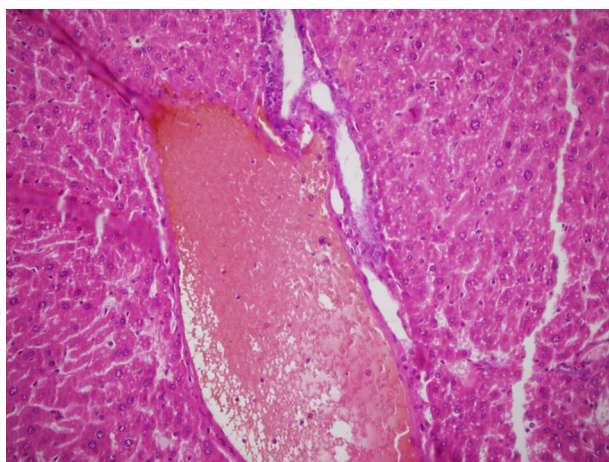
**Table 2.** Data Analysis Using Kruskal-Wallis Method for Evaluating Serum Concentration of Alanine Transaminase, Aspartate Aminotransferase, and Alkaline Phosphatase in Control Groups and Groups Treated With Different Doses of Magnetite Nanoparticles<sup>a</sup>

Groups	Intact Control	Placebo Control	25 µg/gr dose	50 µg/gr dose	75 µg/gr dose	150 µg/gr dose <sup>b</sup>	300 µg/gr dose <sup>b</sup>	P value
<b>ALT, U/lit</b>	34.8500 ± 4.93404	42.0500 ± 2.91051	35.7000 ± 4.94283	35.1500 ± 2.73909	38.4000 ± 3.05045	48.0000 ± 3.92026	69.3500 ± 1.42441	< 0.0001
<b>AST, U/lit</b>	85.9000 ± 6.95020	80.0000 ± 6.19847	79.8500 ± 5.44131	93.4500 ± 4.29780	74.3000 ± 2.86724	195.2000 ± 38.94477	217.9500 ± 6.79377	< 0.0001
<b>ALP, U/lit</b>	76.4500 ± 4.59376	77.8500 ± 6.27673	75.8000 ± 4.66341	77.1500 ± 3.40704	74.8500 ± 3.63137	128.9000 ± 13.67633	146.3500 ± 10.58934	< 0.0001

Abbreviations: ALP, Alkaline Phosphatase; ALT, Alanine Transaminase; AST, Aspartate Aminotransferase.

<sup>a</sup>BALB/c mice treated with 150 and 300 µg/gr doses of Fe<sub>3</sub>O<sub>4</sub>-NPs showed significant difference compared with groups that received lower doses (25, 50, 75 µg/gr) and controls.

<sup>b</sup>Significant difference (P < 0.0001).



**Figure 2.** Venous Congestion of Liver in Mice Treated With 300 µg/gr Doses of Magnetite Nanoparticles

## 5. Discussion

The unique features of NPs, including their specific chemical and physical characteristics in size, shape and high proportion of surface to volume, have made them attractive for use in a variety of medical and biological settings. However, to date, there are few studies directly or indirectly evaluating the toxic effects of these nanomaterials and, presently, there is a lack of clear guidelines to quantify these effects. Among them,  $\text{Fe}_3\text{O}_4$ -NPs, due to their specific magnetic, optical, electrical and chemical properties, are considered for a wide range of therapeutic and biological applications, such as materials for magnetic hyperthermia, as contrast agents in magnetic resonance imaging (12), for cell labeling or for targeted drug delivery (13). The hazards of these particles, as well as their benefits, should be explored urgently to determine a safe dose at which the risks and benefits of  $\text{Fe}_3\text{O}_4$ -NPs application are equivalent. Therefore, the aim of the current study was to determine potential adverse effects of  $\text{Fe}_3\text{O}_4$ -NPs, by evaluating the changes in the level of liver enzymes (AST, ALT and ALP) and histological properties of liver tissue, after exposure to different doses of these NPs. Our results indicated that administration of  $\text{Fe}_3\text{O}_4$ -NPs in 150 and 300 µg/gr doses leads to histopathological effects on liver, including necrosis of hepatocytes, plethora of Kupffer cells, congestion of central venous circulation and hypertrophy of hepatocytes. A significant increase in Kupffer cells and significant decrease in hepatocytes count per unit area were observed in these groups, as well. Meanwhile, evaluating the alteration in hepatic enzymes showed a significant increase in the levels of AST, ALT and ALP, which are common markers for hepatic toxicity. However, administration of lower doses of these nanoparticles (25, 50 and 75 µg/gr) was not associated with significant adverse histological effects on liver and no significant alterations in liver enzymes were detected.

Different available studies have investigated toxicity of IONPs (14, 15). However, the reported results are not consistent with one another. Kim et al. (16) suggested that IONPs do not cause apparent toxicity to mice, in vivo. In contrast, multiple other studies have reported severe adverse effects, associated with exposure to these NPs (17-19). For example, in their study Zhu et al. (17) indicated that administration of  $\text{Fe}_3\text{O}_4$ -NPs induce lung injury, increase microvascular permeability and cause the lysis of lung airways epithelial cells, in mice. The  $\text{Fe}_3\text{O}_4$ -NPs were also shown to enter the central nervous system, induce severe oxidative stress and damage nerve cells in mice (18). A study conducted by Babadi et al. (20) indicated that exposure to 150 µg/kg body weight  $\text{Fe}_3\text{O}_4$ -NPs for 15 days leads to a significant increase in AST, ALT and ALP levels. Factors that can cause these conflicting reports include the physicochemical properties of the NPs, such as size, shape and solubility. In addition, the experimental model, exposure times and concentrations may cause different results (14). Different available studies explore the possible underlying toxicity mechanisms of IONPs. Several reports have suggested contribution of excessive reactive oxygen species (ROS) to most intracellular and in vivo toxicities from IONPs. Another reported mechanism of IONPs induced toxicity is iron accumulation in tissue organ, due to their required high concentration in biomedical application.

The results of our study indicated a dose dependent toxicity of  $\text{Fe}_3\text{O}_4$ -NPs. These experiments helped to determine a recommended tentative low-risk dose of  $\text{Fe}_3\text{O}_4$ -NPs for in vivo systemic delivery. We indicated that, even though 150 and 300 µg/gr doses showed adverse effects on liver, the administration of  $\text{Fe}_3\text{O}_4$ -NPs in doses of 25, 50 and 75 µg/gr can be regarded as safe. However, the number of samples for this study was not high and the duration of exposure was short. Longer treatment periods should be tested with a larger number of animals to confirm that the mentioned doses are safe.

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## Footnote

**Authors' Contribution:** Study concept and design: Kazem Parivar, Fatemeh Malekvand Fard, Mahsa Motavaf; acquisition of data: Fatemeh Malekvand Fard, Mahdieh Bayat; analysis and interpretation of data: Kazem Parivar, Seyed Moayed Alavian; drafting of the manuscript: Mahsa Motavaf, Mahdieh Bayat; critical revision of the manuscript for important intellectual content: Mahsa Motavaf, Seyed Moayed Alavian, Fatemeh Malekvand Fard.

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