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Original Article

Examining the effects of 20 nm nano-iron oxide particles on fetal heart development of NMRI mice

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Abstract

Introduction: Researchers take a great interest in nanoparticles due to their unique properties and high level of performance. Yet, despite the functions of nanoparticles in various sciences and industries, their potential effects on human health especially fetal heart have not been fully investigated. The destructive effect of iron nanoparticles on the fetal heart is inevitable. Therefore, the aim of this study was to investigate the effect of iron oxide nanoparticles on fetal heart growth and development in vivo and in vitro on NMRI mice.

Materials and Methods In this study, mice were divided into three groups: 1- Control: (without the effect of iron oxide), 2- Sham: (injection of solvent iron oxide and distilled water on the 9th day of pregnancy) 3- Treatment: (under the influence of different concentration of nano-iron oxide particles (10, 30, and 50 µg/kg body weight) on the 9th day of pregnancy). On day 16 of pregnancy, fetuses were taken out and their heart was removed (in vivo method) and analyzed by morphological, histological, and statistical criteria. As for in vitro method, pregnant mice were anesthetized on day 15. The embryos were removed from the body. Their hearts were separated and cultured in a culture medium containing a certain dose of iron oxide nanoparticles. Then, morphological and histological changes were examined.

Results: Injection of iron nanoparticles at concentrations of 10, 30, and 50 g/kg caused a significant increase in fetal body weight and height. However, in the results of the examinations on the heart organs, no change in the diameter, weight, wall thickness of the ventricles and atria was observed both macroscopically and microscopically.

Conclusions: In the findings of our study, increase in body length and weight of fetuses can significantly indicate the possibility of increased cell division in the fetus and the ability of these nanoparticles to pass through the placenta and transfer from mother to fetus.

Keywords: Iron Nano Oxide Particles, Heart Development, NMRI Mouse

1. Introduction

Nanotechnology includes the ability to produce new materials, tools, systems by controlling molecules and atoms and using their properties at the nanoscale, which is used in various fields such as pharmacy, medicine, food industry, and the like. Nanotechnology is also the basis of research when it comes to new therapies for a number of human diseases [1]. The goal of nanomedicine formulation is to improve the distribution and accumulation of drugs at target sites [2]. They can help increase the therapeutic index and create treatments with less toxicity and more efficacy [3]. Nanoparticles are highly attractive for studies due to their unique properties such as the ability to bind, adsorb and transport compounds such as drugs, probes, proteins, and also their high functional level [4]. Due to their unique magnetic and physicochemical properties, Fe2O3 and Fe3O4 iron oxide nanoparticles are used to develop magnetic resonance imaging applications such as magnetic resonance imaging, targeted drug delivery, and hyperthermia anti-cancer therapies [5]. However, many scientific studies have investigated the precise mechanisms of transport of iron nanoparticles to target organs and tumors, and also much attention has been paid to the toxic effects of these iron nanoparticles that prevent their use [6]. For example, one study reported that nano-iron oxide particles with a diameter of 20-30 nm caused dose-dependent toxicity in normal human alveolar epithelial cells. The possible effect of toxicity on the easy entry of small nanoparticles into cells has been suggested as the potential cause [7]. Van Den Boss et al. (2003) also showed that Feridet (dextran-coated nanoparticles) can show significant toxic effects on macrophages, including reduced proliferation and cell death [8]. Further studies on the direct cause of toxicity have been shown to be related to oxidative stress and free radicals [9].

In general, the potential toxicity of iron oxide nanoparticles is usually determined by diagnostic tests such as cell viability, cytotoxicity, oxidative stress, inflammatory and genotoxic reactions *in vitro*, as is common with other nanoparticles [10]. If slight or negligible effects on the used concentration are observed in the toxicity test, then samples are submitted for *in vivo* testing in animal models (similar to human conditions) [11]. However, since the toxic response of these nanoparticles *in vitro* cannot be accurately observed *in vivo*, few traceable changes are observed in studies of iron oxide nanoparticles. Due to these limitations and the safety conditions of these nanoparticles, more detailed studies are needed to accurately estimate the toxicity of these nanoparticles on cell function.

The heart is the first organ in the body to develop and function in the fetus [12]. Since nanomaterials are widely used in human life today in various fields as well as in the pharmaceutical and medical industries especially treatment and diagnosis, these nanoparticles can cross the placenta and many mothers may encounter them during pregnancy. Given that, these nanoparticles can have effects on the fetal heart. As a result, these topics led us to investigate the various effects of these nanoparticles on the

fetal heart. The advantage of this study is that it evaluates the effects of nano-iron oxide particles, both *in vitro* and *in vivo*, in different doses on indicators such as fetal weight and length, cardiac growth, heart weight, and diameter, as well as microscopic examinations. This can help scientists with optimal and low-risk use of this nanomaterial in medical sciences and nanomedicine.

2. Materials and Methods

2.1. The Animal under Study and its Preparation for Testing

In this study, 60 NMRI mice were purchased from Pasteur Institute in Tehran and studied in the animal room of the Islamic Azad University, Science and Research Branch. The animals were kept in plastic cages with easy access to water and food, and the temperature in the animal husbandry room was 23 ± 2 °C. The cages of male mice were separated from females, and in this study, adult male and female mice were used. At night, one male and three female mice were placed in a cage. Mating usually took place in the middle of the night and resulted in the formation of a vaginal plug (VP). Mice suspected of being pregnant were placed in separate cages and the time of observation of VP or sperm was considered to be the day zero of pregnancy.

2.2. Preparation of Culture Medium

Growth medium, i.e., liquid DMEM medium purchased from Afshar Laboratory Equipment, was of high glucose type. In the study groups, different doses of nano-iron oxide particles were added to this culture medium. All containers and solutions used in culture are well sterilized to avoid possible contamination. Autoclaved containers were covered with aluminum foil and placed in nylon and sterilized in an autoclave at 2 °C for 2 hours. The solutions used, such as HBSS and DMEM, were sterilized by a Millipore needle filter with a pore diameter of 0.22 microns.

2.3. Preparation of Nano-Iron Oxide Solution and Injection

The nano-iron oxide (Fe2O3) used in this experiment was in the form of reddish-brown powder with about 20 nm diameter and 99.5% purity, purchased from Nano Rahpooyan Mahan company. To prepare the solution, a certain amount of double-distilled water was added to this powder and then it was put in a sonicator for fifteen minutes until the nano-iron oxide particles were completely dissolved in the distilled water, then this liquid was mixed well by a rotary apparatus. After this step, the resulting solution was immediately drawn with an insulin syringe and transferred to the animal room. Mice were weighed before injection to calculate the dose of injection according to weight. After calculating the dose by injection method, it was injected by intraperitoneal (IP) method under sterile conditions.

2.4. Animal Grouping

In this study, 60 pregnant female mice were divided into three groups. 1- Control group: (without the effect of nano-iron oxide particles) 16-day-old embryos were examined. 2- Sham group: On the 9th day of pregnancy, nano-iron oxide solvent, distilled water was injected into the pregnant mother and on the 16th day the fetuses were examined. Treatment group: Pregnant mothers on the 9th day of pregnancy were exposed to different concentrations of iron oxide nanoparticles of 10, 30, and 50 micrograms per kilogram of body weight and on the 16th day the fetuses were examined.

2.5. General Steps of in vivo Method

On the 16th day of pregnancy, pregnant mice were anesthetized with ether or chloroform and transferred to the dissection pan. When dissecting the mice, they were laid on their backs, and after applying alcohol on the skin of the abdomen, a longitudinal incision was

made in the abdomen and the skin of the abdomen was removed. The uterine tubes were transferred to a petri dish containing HBSS after rinsing slightly in physiological saline, and then the embryos were removed from the uterus and amniotic membrane under a stereomicroscop. After weighing and measuring the fetus and heart with a caliper, they were transferred to containers containing fresh HBSS to prevent tissue damage. The heart tissue was separated under the laminar flow cabinet and placed in separate containers, with a Bouin fixator added to fix the tissues. After stabilization, dehydration was performed with ascending degrees of ethyl alcohol, which includes 70% (for 1-2 hours), 90% (until the day of molding), and 100% (1 hour), respectively. Then clarification was performed by toluene for 1 hour and then a paraffin bath with a melting temperature of 57-60 degrees was performed for one hour. Finally, the samples were placed in molds with a specific orientation and immersed in paraffin. The cooled paraffin molds were transferred to the microtome for cutting, and 5 μm thick slices were prepared and placed on the slides and left at laboratory temperature overnight until the slices adhered completely to the slide. Then hematoxylin-eosin staining was performed.

2.6. in Vitro Culture Steps and Practical Experience

After taking the above steps and separating the heart tissue, the hearts were quickly transferred to culture vessels containing a grade for organ culture. First, the culture medium used, DMEM medium was purchased in liquid form from Afshar laboratory equipment, which was of high glucose type. In the experimental groups used, different doses of iron nanoxide were added to this culture medium. After completing the previous steps *in vivo*, hearts were moved on the organ culture grids without damage and in the phase between culture media containing 90% DMEM, 10% FBS, and diluted nono-iron oxide in double-distilled water were incubated in a CO2 incubator with 95% air at 37 °C for one day. Then, under the hood, it was transferred to glass containers and a Bowen fixative was added. Afterwards, the tissues were washed with distilled water and sent to prepare tissue sections and staining. The slides prepared by this method were examined under a light microscope.

2.7. Statistical Analysis

The obtained results were analyzed using SPSS 18 software in $P < .05$, considering standard deviation (SE) and also using One-way analysis of variance (one-way ANOVA) and Tukey test were analyzed and then histograms were drawn using Excel software.

3. Results

3.1. The Effect of Iron Oxide Nanoparticles on Fetal Weight and Length *in vivo*:

Fetal weight on day 16 in the third experiment group (receiving iron oxide particles of 50 μg concentration) had a significant increase compared to the sham and control groups, but the other experimental groups did not increase significantly. Also, fetal length in the third experiment group had a significant increase compared to the control and sham groups and the other groups did not show a significant difference (Table 1).

3.2. The Effect of Iron Oxide Nanoparticles on Heart Development *in vivo*:

Hearts were isolated from the embryonic body on day 16 of embryonic development and five groups of control, sham, and experiment groups 1 (dose 10), 2 (dose 30), and 3 (dose 50 g/kg µ mouse weight) were evaluated by microscopic, macroscopic, and histologic examination. Macroscopic examination of the heart organ in all five groups of control, sham, and experiments showed that this organ did not undergo any morphological changes (Figure 1).

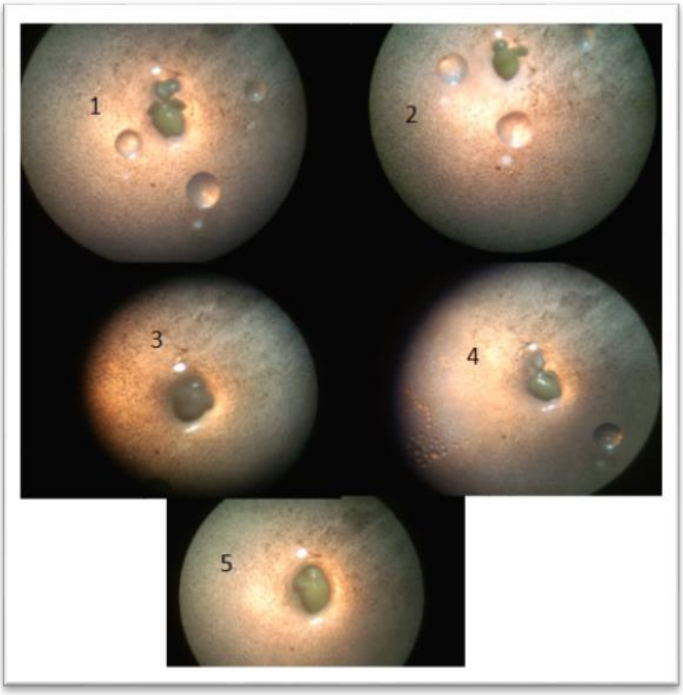


Figure 1. Comparison of morphology and heart size of 16-day-old embryos under an inverted microscope.

3.3. *in vivo* Weight and Diameter Measurement of the Heart

Results demonstrate that heart weight in samples of experiment 1, experiment 2, and experiment 3 compared to control and sham samples did not show a statistically significant increase (P >.05). Total heart length was measured using a vernier caliper. Total heart length in samples of group experiment 1, experiment 2, and experiment 3 in comparison with control and sham samples did not increase statistically significantly (P >.05) (Table 2).

3.4. Microscopic Examinations of Heart Diameter, Atrial and Ventricular Wall Thickness *in vivo*

All tissue sections prepared from fetal hearts were stained on day 16 by the hematoxylin-eosin method and examined under a light microscope. According to microscopic observations, total heart diameter, atrial and ventricular thickness in group samples of experiments 1-3 were not statistically significant compared to control and sham samples (P >.05) (Table 3) (Fig. 2).

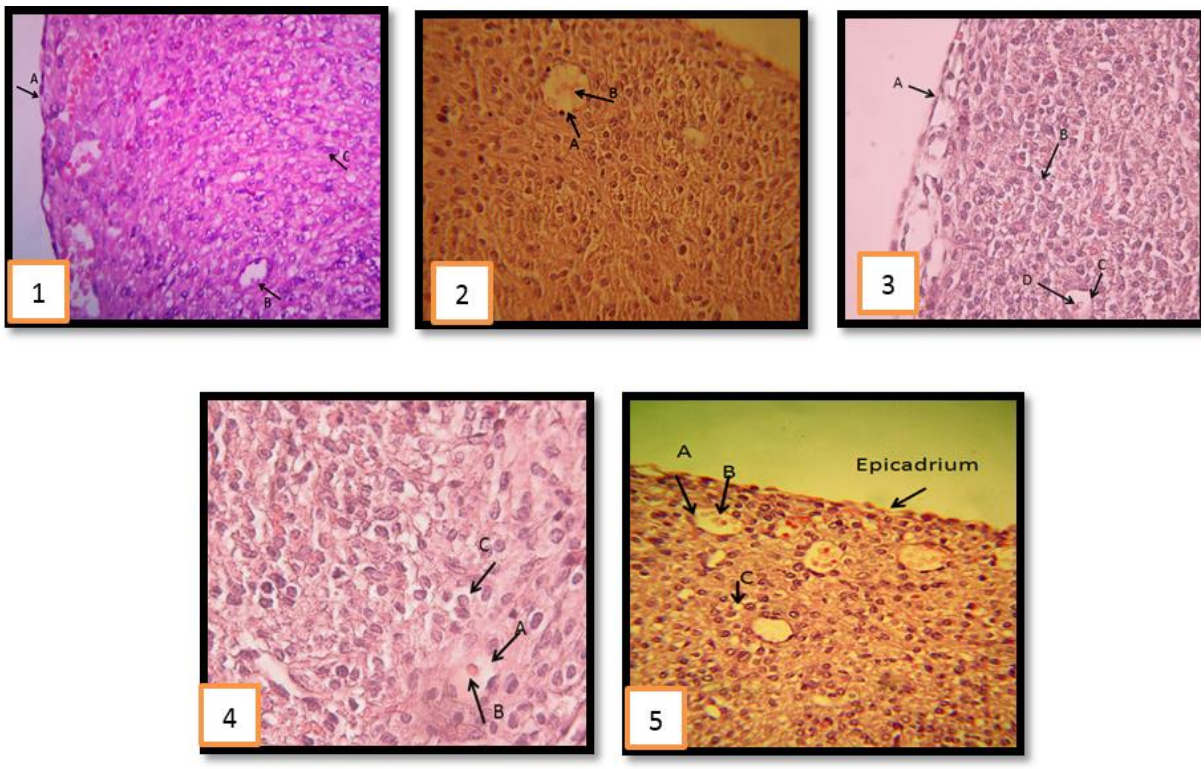
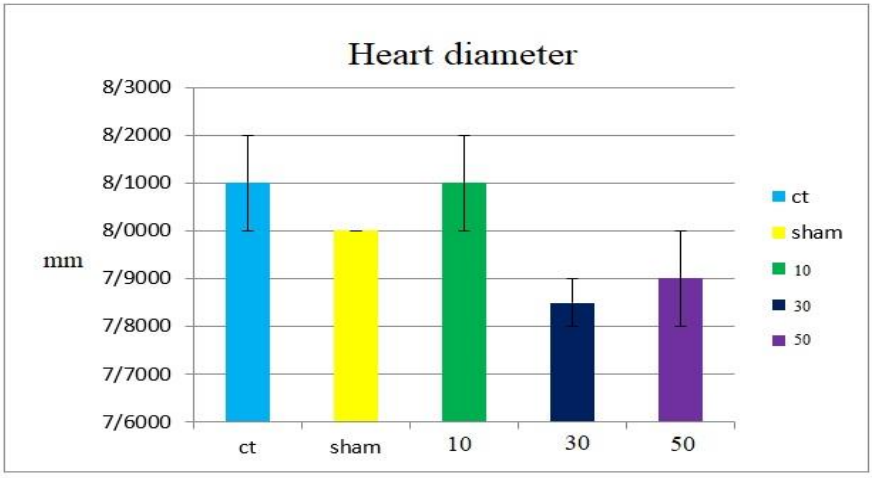


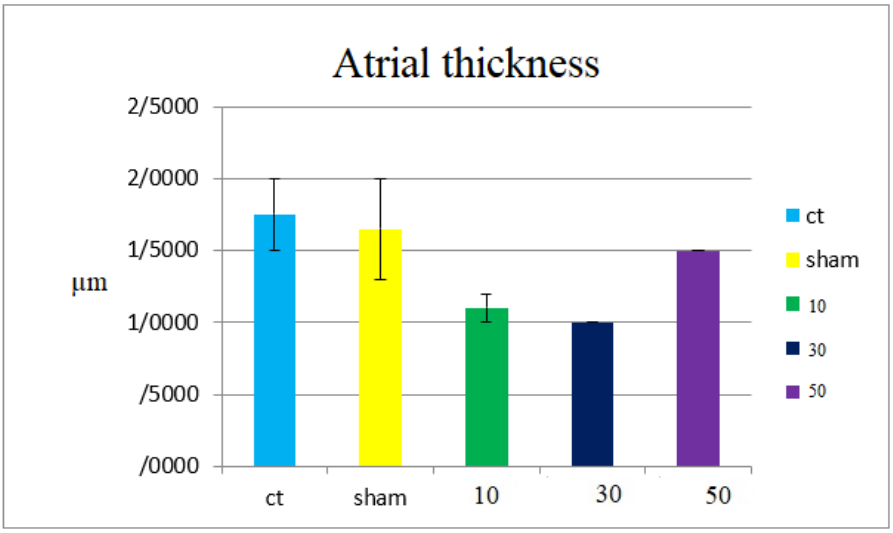
Figure 2. Photomicrograph of the fetal heart from the **1**: control group On day 16(A: Epicardium, arrow B: Capillary, arrow C: heart cells) ,**2**: sham group(A: Capillary, arrow B: Red blood cell 400X) ,**3** : in experiment group 1 (10 µg/kg)(A: Epicardium, B: Vacuolated heart cell, C: Capillary, D: Red blood cell 400X) ,**4**: experiment group 2(30 µg/kg)(A: Capillary, B: Red blood cell, C: Vacuolated cell 400X) ,**5**: experiment group 3 (50 µg/kg) (A: Capillary, B: Red blood cell, C: Vacuolated cell and epicardium 40X).

3.5. Evaluating Heart Diameter, Atrial and Ventricular Thickness *in vitro*

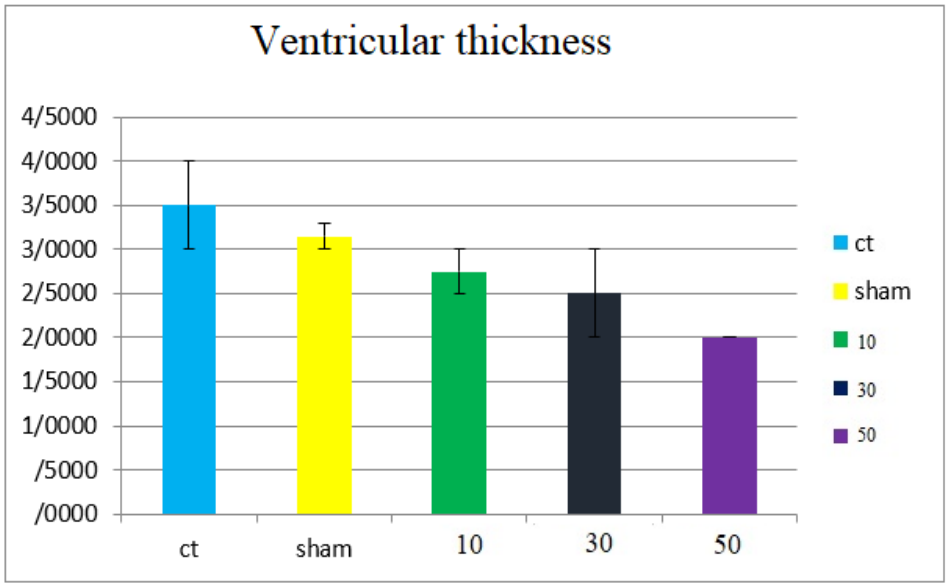
Studies under *in vivo* conditions show that diameter of heart and atrial and ventricular thickness in samples of experiment 1-3 in comparison with samples of sham and control have not shown significant differences (Graph 1-3)



Graph 1. Comparison of heart diameter on 16-day-old fetuses across control, sham, experiment 1 (10 µg/kg), experiment 2 (30 µg/kg), and experiment 3 (50 µg/kg) *in vitro*



Graph 2. Comparison of atrial wall thickness of 16-day-old fetuses across control, sham, experiment 1 (10 µg/kg), experiment 2 (30 µg/kg), and experiment 3 (50 µg/kg) *in vitro*



Graph 3. Comparison of ventricular wall thickness of 16-day-old fetuses across control, sham, experiment 1 (10 µg/kg), experiment 2 (30 µg/kg), and experiment 3 (50 µg/kg) *in vitro*

3.6. Microscopic Results of the Effect of Iron Oxide Nanoparticles *in vitro*

Hearts were isolated from embryonic bodies on day 15 of embryonic development and prepared for culture, and 24 hours after placement in the culture medium (i.e., the 16th day of embryonic development of the heart) the groups of control, sham, and experiments 1-3, underwent macroscopic and microscopic examinations in which no morphological abnormalities were observed and all hearts were healthy (Figures 6 to 10).

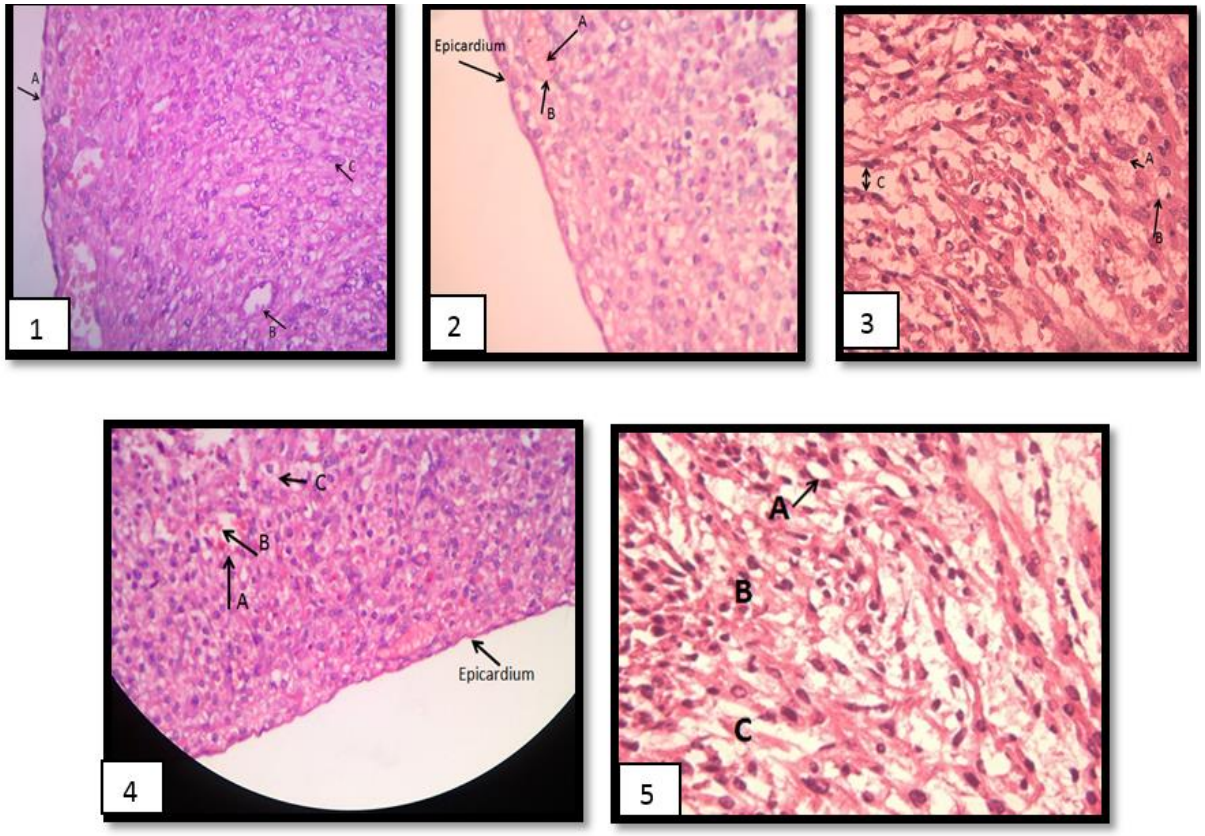


Figure3. Photomicrograph of fetal heart from, **1**: control group on day 16.(A: Epicardium, B: Capillary, C: Intact heart cells), **2**: sham group on day 16(A: Red blood cell, B. Capillary, epicardium), **3**: experiment 1 group on day 16 (10 µg/kg)(A: Intact heart cells, B: Vacuolated heart cells, C: Cell gaps),4: experiment 2 group on day 16 (30 µg/kg)(A: Capillary, B: Red blood cell, C. Vacuolated cell), **5**: experiment 3 group on day 16 (50 µg/kg)(A: Vacuolated heart cells, B: Abnormal shape of cells, nuclei, and necrosis, C: Cell disorganization and increment of cells gaps) (400X) (H&E staining).

4. Discussion

Recent advances in nanotechnology indicate that iron oxide nanoparticles not only are used and predicted in a variety of fields from catalysis and optoelectronics to sensors, environmental remediation, and biomedicine, but also will continue at higher speed in the future [13]. It seems that reducing their size increases the specific surface area of nanoparticles, which is a key factor in the performance of catalysts and structures such as electrodes or the efficiency of technologies such as fuel cells and batteries [14]. Despite the numerous benefits of nanoparticles, several studies have reported toxicity, free radical production, fibrosis, inflammation, and tumor formation in various nanoparticles including iron and zinc [15]. Toxic effects of iron oxide nanoparticles including liver damage and toxicity and increased concentrations of liver enzymes, testicular tissue damage, and the effect on sex hormones by zinc oxide nanoparticles have been reported [16,17]. Therefore, it is necessary to investigate the safety-related issues and attendant risks. Iron, as an abundant metal in body, plays a vital role in living organisms by participating in various processes, including oxygen transfer, DNA synthesis, and electron transfer. Yet, the oxygen concentration in the body must be fully regulated because excessive concentrations of iron can lead to tissue damage by forming free radicals. Disorders of iron metabolism among a wide range of common human diseases include a wide range such as anemia and degenerative neurological diseases [18]. In this study, the effect of iron nanoparticles on mouse embryos on day 16 of pregnancy was well demonstrated. These nanoparticles entered the fetus through the placenta and increase in dose caused a significant increase in body length and fetal body weight. However, no significant change was observed in the weight and diameter of the heart as well as the wall thickness of the atria and ventricles. In this study, no mortality and severe complications were seen in the mother and fetus after injection, and no congenital anomalies were observed.

Studies on the biocompatibility of iron nanoparticles have reported that these nanoparticles can be effectively absorbed by human endothelial cells *in vitro*. Further *in vivo* studies have shown that although iron nanoparticles are excreted very rapidly in the urine, they can lead to toxicity to the liver, kidneys, and lungs while the brain and heart remain unaffected, which is consistent with the findings of the present study. It has also been shown that coating with iron nanoparticles can have a significant effect on increasing cell entry [1]. Lasagna-Reeves et al. (2010) also showed that most of the nanoparticles are removed by the liver and spleen after administration and entering the bloodstream, and other tissues of the body, including the kidneys and heart, are in the next degree in terms of tissue accumulation of metal nanoparticles. The mechanism of toxicity of metal nanoparticles depends on the interaction of nanoparticles with biomolecules, producing reactive oxygen species and inducing oxidative stress [19]. Macroscopic findings of our study also showed no morphological changes in the heart organ, which could be due to fewer iron oxide particles entering the heart tissue than other organs.

In the present study, magnetic iron oxide nanoparticles with a diameter of 20 nm were used. Macroscopic changes in the fetus showed the ability of these nanoparticles to pass through the placenta and transfer from the mother to the fetus. In the results of our studies, a significant increase in the length and weight of the embryos was observed, which is probably due to the increase in cell divisions. In 2011, Chatterjee et al. examined the effect of iron oxide nanoparticles on cell growth of E. coli and found that iron oxide nanoparticles increased cell divisions, and a sudden increase in bacterial cell length was seen in their experiments [20]. A study conducted in 2009 with the purpose of investigating the effect of ferucarbotran, a type of nano-iron oxide particle, on mesenchymal stem cells, Hung et al. came to the conclusion that the substance enhances cell growth and cell cycle progression through altering the expression of regulatory proteins and reduction of intracellular hydrogen peroxide (H2O2) [21]. In another study, the toxicity of magnetic nanoparticles by injection in mice was studied and the results showed a safe potential of these nanoparticles with no death being reported. No side effects were seen; histopathological examination did not show the presence of iron in macrophages; and tissue changes were not seen in major organs such as the liver, lungs, spleen, brain, and heart [22]. In the findings of the present study, no mortality and severe complications were observed in the mother and fetus after injection, and no congenital anomalies were observed in the fetuses. In a study on the toxicity of carboxyethylsilanetriol (CES) coated with iron oxide nanoparticles on different cell lines of different origins including brain, lung, heart, liver, kidney, skin, colon, and cervix, the researchers reported that the same concentration of these nanoparticles coated with iron oxide had a significant toxic effect on glial and nerve cells derived from the brain and lung cells, but showed less toxicity in other tissues including the heart. Concentrations of 2 mM of these nanoparticles in neurons and the brain reduced cell viability, and a concentration of 4 mM reduced cell viability by more than 80% in glial cells. The results of this study indicate that the degree of toxicity of nanoparticles depends on their dose as well as cell type [23]. In the findings of the present study, increasing the concentration of nano-iron oxide particles was more toxic (experiment group 3). In a study to evaluate the toxicity of magnetic iron oxide nanoparticles in different tissues *in vitro*, Vakili et al. (2020) reported that there is a lot of evidence that the toxicity of iron nanoparticles depends on the particle size, dose, and level of change, as well as the type of exposed cell [24]. In another study, a single dose of intravenous injection (.8 mg/kg) of iron oxide nanoparticles in rats (*in vivo*) was reported to have toxic effects on the liver, lung, and kidney while the heart and brain remained unaffected [25], which is consistent with the findings of the present study.

5. Conclusion

Despite the numerous benefits of nanoparticles and their use in various medical fields such as diagnosis and treatment, there are serious and important problems related to their toxicity that need to be extensively studied. Our research has been carried out in three doses of 10, 30, and

50 µg/kg of iron oxide nanoparticles with a diameter of 20 nm in both *in vivo* and *in vitro* environments. Weight and length of fetuses had a significant increase and a dose-dependent relation that suggests dose-dependent toxicity *in vivo*, as well as the ability of these nanoparticles to pass the placenta. However, no significant difference was observed in the diameter and weight of the heart as well as the thickness of the walls, indicating that these nanoparticles spare heart tissue.

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Conflict of interest

The authors declare no conflict of interest.

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Criteria/ Group	Control	Sham	E1	E2	E3
Fetus weight (mg)	0.749±0.0635	0.744±0.0261	0.794±0.0421	0.910±0.0311	**0.984±0.0411
Fetus length (mm)	17.77±0.633	17.76±0.400	18.8±0.296	19.10±0.366	**19.92±0.229

Table 1. Results of comparison of fetal weight and length across control, sham, and experiment groups 1-3 ($\bar{x} \pm SE$)($** \ P < 0.01$)

Criteria / Group	Control	Sham	E1	E2	E3
Heart weight (mg)	0.0096± 0.0004	0.0096± 0.0002	0.0096± 0.0003	0.0097± 0.0004	0.0098± 0.0004
Heart diameter (mm)	2.577± 0.0458	2.567± 0.0451	2.633± 0.0576	2.642± 0.0553	2.661± 0.0261

Table 2. Results of statistical analysis across groups of control, sham, and experiment 1-3

($\bar{X} \pm SE$)

Criteria / Group	Control	Sham	E1	E2	E3
Heart diameter (mm)	7.900±0.360	7.833±0.1666	7.366±0.5783	6.733±0.6227	8.366±0.2333
Atrial thickness	2.000±0.360	1.9000± 0.10000	2.000±0.2886	1.166±0.1666	2.000±0.2886
Ventricular thickness	2.166± 0.1666	2.000± 0.0000	2.8333± 0.1666	2.3333± 0.1666	2.6660± 0.600

Table 3. Results of statistical analysis of heart size, atrial and ventricular thickness across groups of control, sham, and experiment 1-3 ($\bar{X}$ ±SE)