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Full Length Research Paper

Effect of magnetic iron oxide nanoparticles on pregnancy and testicular development of mice

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In this study, considering the high sensitivity of developing fetal organs, different doses of iron oxide nanoparticles coated with dimercaptosuccinic acid (DMSA) were injected intraperitoneally to pregnant mice. The magnetic and structural properties of DMSA-coated nanoparticles were examined by Alternating Gradient-Force Magnetometry, X-Ray Diffraction and Fourier Transform Infrared Spectroscopy. The histological studies of the fetal liver and placenta sections showed presence of nanoparticles in these organ systems. Weight change and the number of pups born by pregnant mice in comparison with controls were not significantly different. But, a significant decrease was seen in infants growth from the mothers treated with doses higher than 50 mg/kg. The testicular histological studies of these infants showed decrease in spermatogonia, spermatocytes, spermatids and mature sperm significantly. Although, some studies revealed the nontoxic effect of iron oxide nanoparticles in adult mice, the present study indicated that, the doses higher than 50 mg/kg of DMSA-coated magnetic nanoparticles can disrupt embryo development.

Key words: Magnetic nanoparticles, pregnancy, testicular development, toxicity.

INTRODUCTION

Nanoparticles have very specific chemical and physical characteristics in their size, shape and high proportion of surface to volume. These characteristics have made them appropriate to be used in many medical and biological cases (Berry and Curtis, 2003; Salata, 2004). Nowadays, in order to increase the effectiveness of nanoparticles in biological systems, different materials, such as albumin, dextran (Berry et al., 2003; Lacava et al., 2001), polyethylene glycol (Gupta and Curtis, 2004), polyethylene oxide (Thunemann et al., 2006) and aspartic acid (Sadeghiani et al., 2005), have been used to coat nanoparticles surface. Presence of such coatings helps the stability of nanoparticles in blood circulation and tissues, and also decrease of their toxic effects (Shubayev et al., 2009). In physiological conditions, magnetic nanoparticles coated with organic molecules are demonstrated to create stable biocompatible magnetic colloids (Chen et al., 2008). There are extensive researches on the application of iron oxide nanostructures in medicine, such as tumor therapy, magnetic resonance imaging (MRI), drug and gene transfer to cells and labeling of macromolecules and cells (Salata, 2004). However, little is known about the nanoparticles toxicity or side effects on cells and tissues, particularly under *in vivo* conditions. Moreover, limited reports conducted on these aspects have had controversial results, for example, a recent study suggested non-toxicity of iron oxide nanoparticles under *in vivo* conditions (Kim et al., 2006), whereas, some others have reported minimal toxicity or severe cell death (Hafeli and Pauer, 1999; Garcia et al., 2005, Shubayev et al., 2009). Because of the higher sensitivity of tissues and organs in the early stages of embryonic

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Abbreviations: DMSA, dimercaptosuccinic acid; **XRD,** X-ray diffraction; **FTIR,** fourier transform infrared spectroscopy; **AGFM,** alternating gradient force magnetometry.

development to external factors (Takeda et al., 2009), the present study was undertaken to investigate side effects and tissue distribution of DMSA-coated Fe_3O_4 nanoparticles in pregnant mice and their new born pups.

MATERIALS AND METHODS

Methods of producing iron oxide

DMSA-coated Fe_3O_4 nanostructure was prepared according to the method of Pisanic et al. (2007). Briefly, three starting solutions were made by adding the 1:2:8 proportion of $FeCl_2$, $FeCl_3$ and NaOH (Merck Co.) to deionized water separately, under atmosphere condition of N_2 and vigorously stirred. In the construction process, DMSA (0.01 M, dimercaptosuccinic acid, $C_4S_2O_4H_6$, Aldrich Chemical) was prepared in deoxygenated deionized water and added via nitrogen bubbling to the magnetic stirrer solution. The resulting mixture was washed by deionized water and centrifuged**.** The precipitate was dried at 40°C and the chemical interaction between Fe_3O_4 and DMSA was investigated by Fourier Transform Infrared Spectroscopy (FTIR, JASCO FT/IR-680 PLUS). The magnetic properties and spinal structure of the preparation was examined by Alternating Gradient-Force Magnetometry (AGFM, Lake Shore) and X-Ray Diffraction (XRD) using Bruker D_8 ADVANCE (λ=0.154nm Cu K_α) radiation .

Breeding animals and treatments

A number of male and female mice of Balb/C strain were purchased from RAZI Vaccine and Serum Research Institute (Hesarak, Iran). The animal studies were performed in accordance with regulatory guidance on the care and use of experimental animals. The animals were kept in 22 to 24°C temperature and natural light for proliferation and studying their life cycle. Twelve virgin mice (3 months old), were selected with a male mice in each treatment and control groups. DMSA-coated Fe_3O_4 nanoparticles with doses of 50, 100, 200 and 300 mg/kg were prepared in saline and single-dose injected intraperitoneally to the pregnant mice on gestation day 8, because the blood-placenta barrier and gonad development begin after 5 to 7 days after gestation. Gestation begins with the sign of a vaginal plug as evidence of copulation or gestation day 0. Only saline was injected to the control group. The body weights of animals were measured and recorded until gestation day 19. The number and weight of the born infants and the length of gestation were examined and 10 offsprings in any group were weighed 50 days after birth and were compared with the control group.

Detection of iron oxide nanoparticles in the fetal and placental tissues

For this purpose, a number of mice in their day 13 of gestation were dissected under mild anesthesia. Because the liver is the center of drug metabolizing enzymes, we examined liver sections for the presence of the nanoparticles in the fetus. The tissue sections were prepared from fetal liver and placenta. Then, the prepared tissues were stained according to the method described by Garcia et al. (2005), using specific iron Prussian blue method. Accumulation of iron oxide nanoparticles were examined in the cells and tissues as dark blue grains under light microscope.

Histological study of testis

Some 60 to 70 days male offsprings in both control and treatment groups were dissected under mild anesthesia. The testes were removed and immersion-fixed in the fixative (formaldehyde). Tissue sections (5 µm) were prepared after dehydration and were embedded in paraffin. The sections were stained with hematoxilin and eosin and subsequently processed for histopathological examination under light microscope. The morphological structure of seminiferous tubules and mean number of spermatogonia, spermatocytes, spermatids and mature sperms in the tubules were studied.

Statistical analysis

Weight changes of female mice during pregnancy in each group were compared by analysis of variance (ANOVA) test (Repeated Measure ANOVA) and comparing the mean weight of mothers, length of gestation, weight and number of infants and the number of cells in semini-ferous tubules using one-way ANOVA and t-test by SPSS (version 15) computer program.

RESULTS

The results of XRD, FTIR and AGFM

The spinel structure was synthesized clearly by XRD peaks corresponded to the two samples of Fe_3O_4 and DMSA-coated Fe_3O_4 and fined pure nanoparticles with the same phase, respectively. The peaks of corer shell spinel ferrite have broader in band width, in coated nanoparticles with respect to non DMSA-coated Fe_3O_4. From the peak width, a particle diameter of 3 to 9 nm was obtained according to the Scherrer formula (Figure 1).

In the IR spectra b (Figure 2), 3 absorption bands were observed at 1619, 1376 and 576cm^{-1}, the former two bands being assigned to the asymmetric and symmetric stretch of carboxylate (COO^-) of DMSA, respectively. This indicates that, DMSA has been bound to the surface of Fe_3O_4 nanoparticles (Silverstein et al., 1999). Also, the intensity reduction of the DMSA-coated Fe_3O_4 band at 576cm^{-1} (spectrum b) when compared with the Fe_3O_4 band at 581cm^{-1} (spectrum a) showed that, DMSA irreversibly absorbed on the surface of Fe_3O_4 nanoparticles (Lee et al., 1996). The results of the alternating gradient force magnetometry (AGFM) (Figure 3) showed that, the saturation magnetization value (M_S) for Fe_3O_4 is 90 emu/g, while the M_S for DMSA-coated Fe_3O_4 decreased to 32 emu/g.

Results of Prussian blue staining

Figure 4 displays aggregated iron oxide nanoparticles in the intervillous spaces and chorionic villi of the placental tissue sections in day 13 of gestation. Interestingly, these nanoparticles were detected in the sinusoids and heaptocytes of the liver tissue of dissected fetuses (Figure 5). These results indicate that, the DMSA-coated Fe_3O_4 nanoparticles were passed through the membrane of different cells and even blood-placenta barrier and enter liver of the developing fetus.

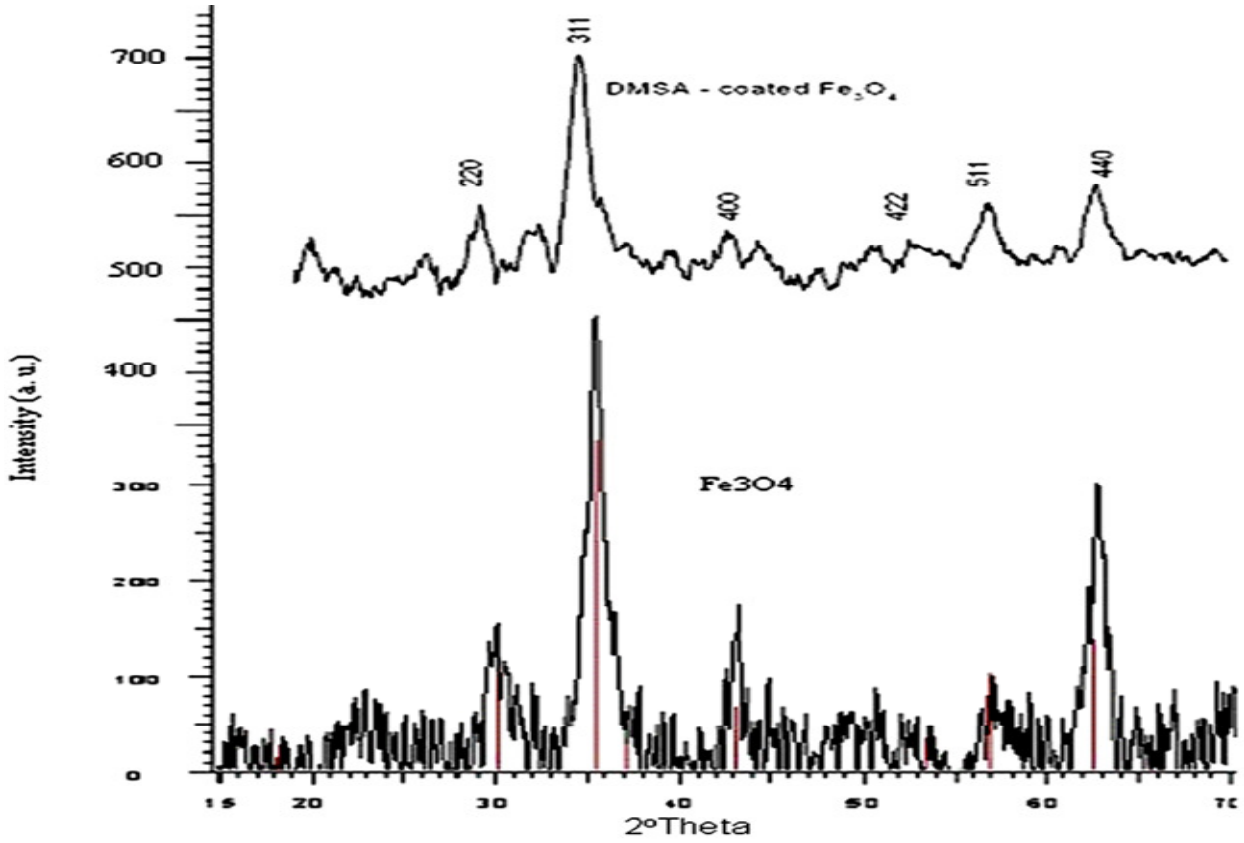

Figure 1. X-ray diffraction patterns of Fe_3O_4 and DMSA-coated Fe_3O_4 (a.u. unit).

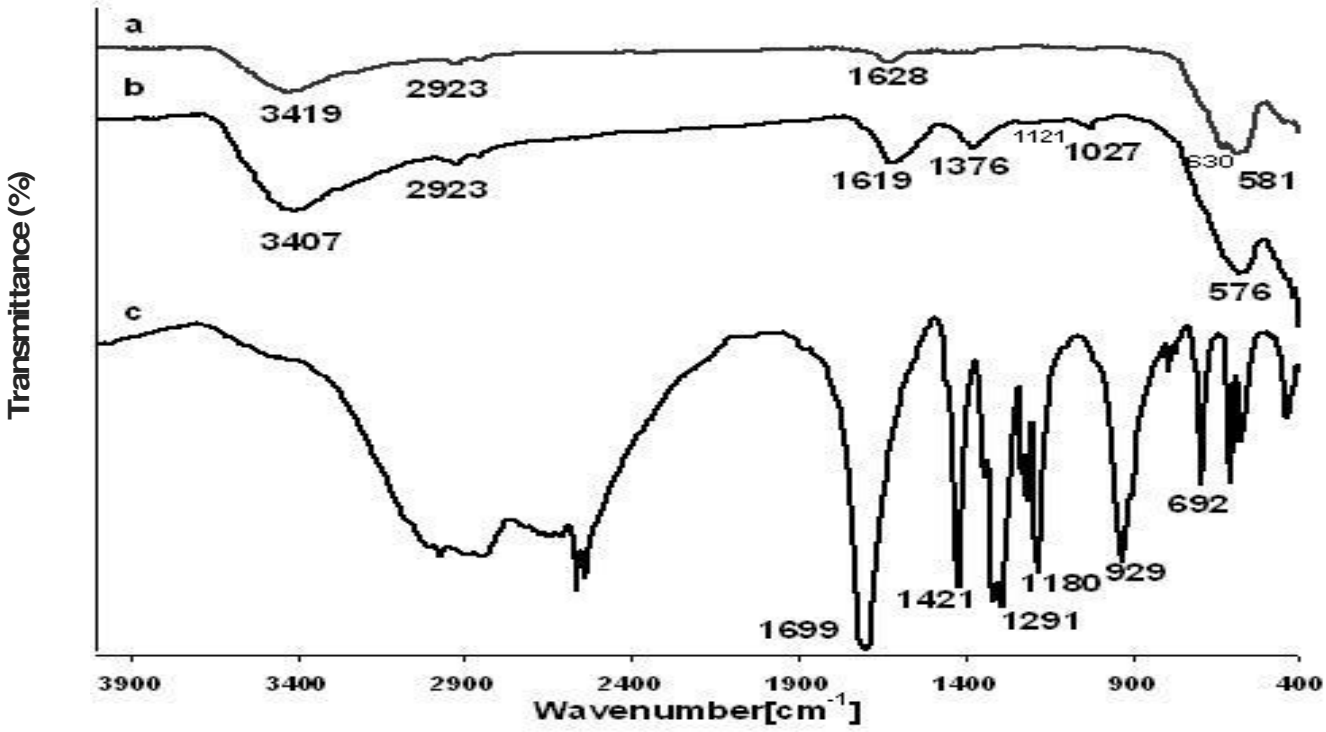

Figure 2. FTIR spectra of (a) Fe_3O_4, (b) @DMSA-coated Fe_3O_4 and (c) DMSA.

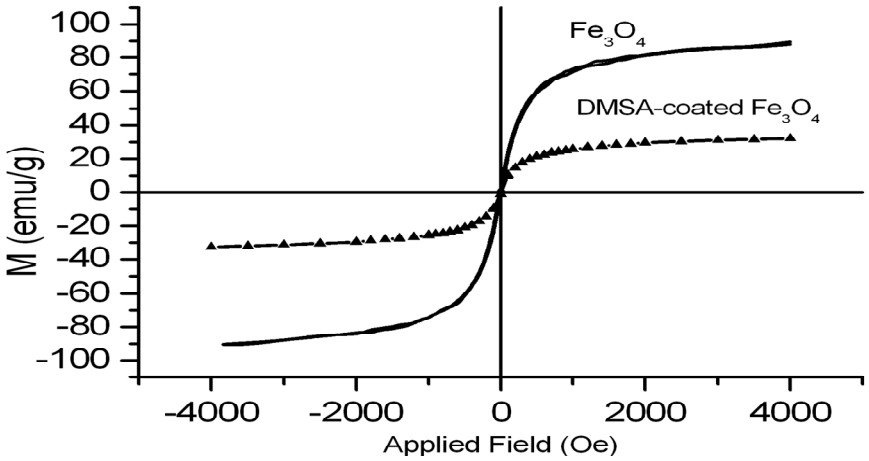

Figure 3. Hysteresis loops at room temperature for Fe_3O_4 and DMSA-coated Fe_3O_4. M, magnetization saturation; Oe, oersted.

The results of weight changes and the number of infants

The pregnant mice were weighed at the time of injection and 4 times after that until the gestation day 19. The length of gestation and number of their pups were counted and weighed after delivery. We recovered an average of 5 infants in each group and the likely fetus resorption rate was not examined. Also, the weight of 10 offsprings of each group at age of 50 days was measured (Table 1). Repeated measure ANOVA showed that, the mean weight of mice in each group at 5 different times of gestation days 8, 10, 13, 16 and 19 days, have not been the same and it has normally increased (comparison of the weights 1 and 5, Table 1, $F = 419.120$, $P < 0.001$). Also, t-test showed significant differences between the mean of all weight groups except weight 1 and weight 2 (equivalent to gestation days 8 and 10), which indicates the increase in weight of pregnant mice or the fetal growth.

One-way ANOVA showed that, the mean weight of pregnant mice at different gestation days, the mean length of gestation ($F = 0.448$, $P > 0.05$), the mean number ($F = 0.672$, $P > 0.05$) and weight of infants ($F = 0.235$, $P > 0.05$) with different groups were not significantly different. It appears that, weights gain by pregnant animals have not been influenced by different concentrations of nanoparticles. In other words, iron oxide nanoparticles injection to pregnant mice has not caused any disorder in pregnancy and fetal growth apparently.

Even though iron oxide nanoparticles injection had apparently no effect on the growth, weight and number of the fetus, the mean weight of 50 day old offsprings in the treatment group, except the dose of 50 mg/kg, were significantly lower (Table 1 *$P < 0.001$, $F = 86.447$) and almost 70% of them died before reaching puberty (the results not shown). In the dose of 50 mg/kg, offsprings' weight, growth and maturity were not significantly different from the control group.

In addition, treated pregnant mice had no behavioral and weight disorder after delivery, during breast feeding and about three months after the injection, in comparison with the control group.

Histological study of testis tissue

Table 2 shows the significant reductions ($P < 0.001$, one-way ANOVA) of mean number of spermatogonia, spermatocytes I and spermatids in testes of 60 to 70 day old offsprings, indicating slow and abnormal growth (the groups of higher than 50 mg/kg), when compared to that of control group. Figure 6 shows, the disrupted spermatogenic cycle and the abnormal development of seminiferous tubules and the noticeable decrease of mature sperms in testis tissue sections of such offsprings.

DISCUSSION

Dimercaptosuccinic acid (DMSA) is a nontoxic chelating agent that nowadays is injected to patients to absorb additional elements in their body. Using this substance, an anionic coating is produced on the nanoparticles' surface that prevents opsonization (accumulation of blood proteins around the nanoparticles) and their removal by reticuloendothelial system of liver and spleen (Garcia et al., 2005). Presence of this substance on nanoparticles surface increases the cell absorption and tissue distribution of nanoparticles and decreases their direct contact with the cells and cellular components and therefore reduces their toxic effects (Pisanic et al., 2007).

The results of XRD, AGFM and FTIR (Figures 1 and 2) indicated that, spinel structure and crystallite size of

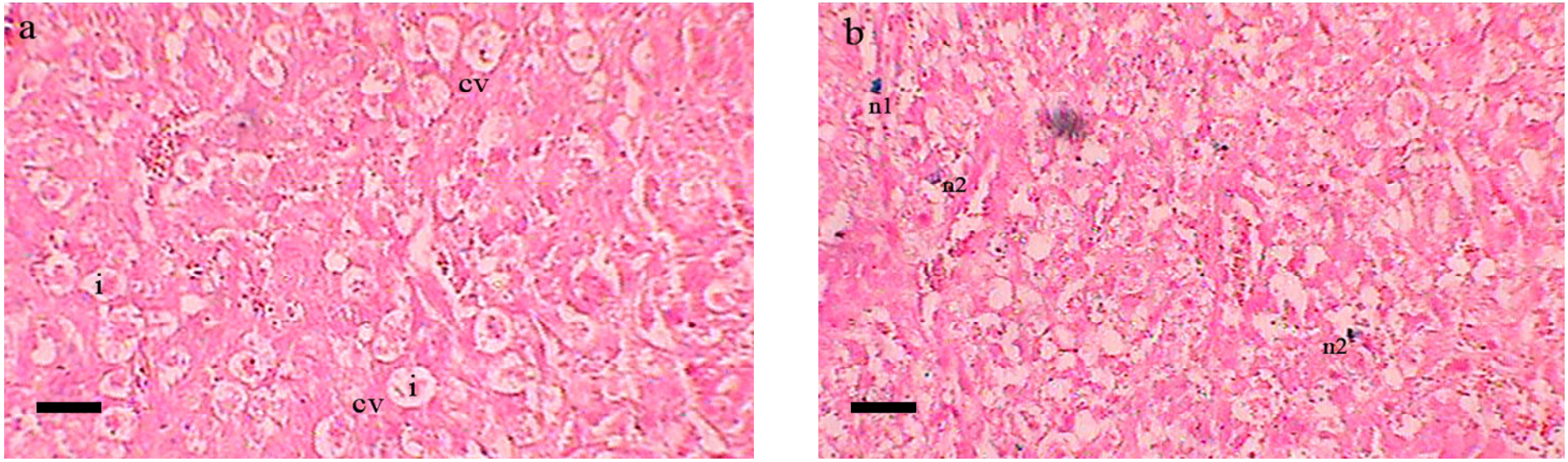

Figure 4. The placenta tissue sections (Perl's Method, ×400) of 13-day-old fetus. (a) Control group and (b) treated group. n1 and n2 show the accumulation of blue iron oxide nanoparticles in the chorionic villi and intervillous spaces respectively. (i. intervillous spaces, cv. chorionic villi).

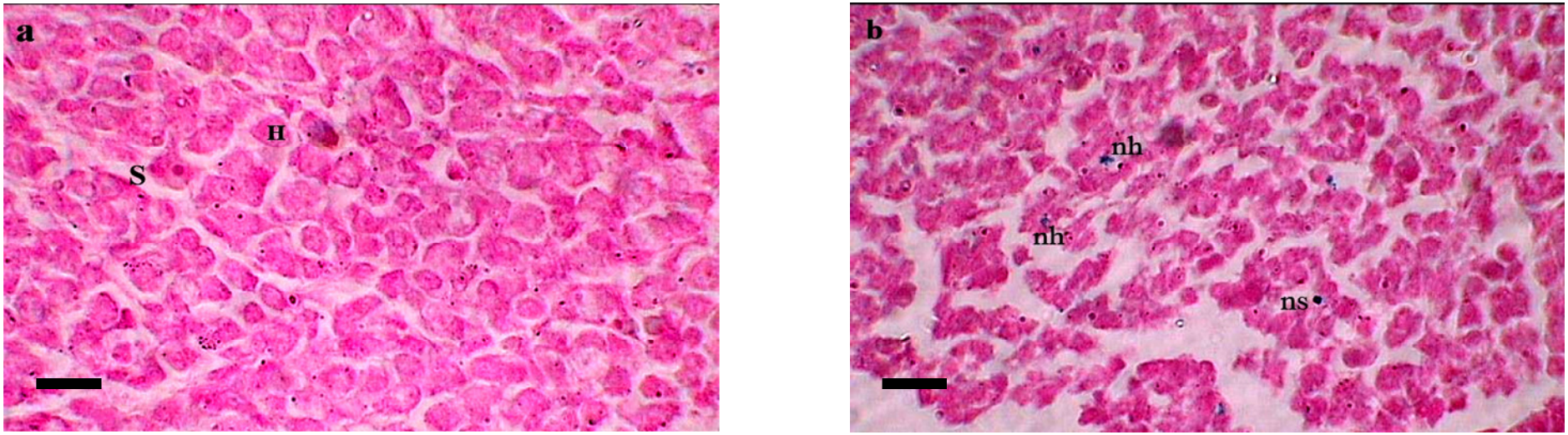

Figure 5. The liver tissue sections (Perl's method, ×400) of 13 day old fetus. (a) Control group; (b) treated group. ns and nh show the accumulation of blue iron oxide nanoparticles in the sinusoids and hepatocytes, respectively (b). (S. sinusoid, H. hepatocyte.

Table 1. Mean weight in different gestation days, length of gestation, weight and number of infants and weight of 50-day-old offsprings in different dose groups.

Parameter	Groups (n=12)				
	Control	50 mg/kg	100 mg/kg	200 mg/kg	300 mg/kg
Mean weight@ (8)§	25.41 ± 1.78	25.25 ± 1.71	26.37 ± 3.76	26.04 ± 2.37	25.66 ± 2.46
Mean weight (10)	24.08 ± 1.79	24.00 ± 1.75	26.62 ± 4.14	25.70 ± 2.28	26.08 ± 3.35
Mean weight (13)	28.45 ± 2.07	27.91 ± 2.33	29.00 ± 3.87	26.79 ± 3.75	30.45 ± 3.15
Mean weight (16)	33.37 ± 1.72	33.16 ± 1.74	32.20 ± 4.02	32.00 ± 4.85	35.41 ± 2.99
Mean weight (19)	38.45 ± 2.83	38.16 ± 2.75	39.45 ± 3.75	37.25 ± 4.15	40.00 ± 2.88
Mean length of gestation	21.12 ± 0.15	21.18 ± 0.12	20.80 ± 0.85	20.95 ± 0.45	21.19 ± 0.25
Mean weight of the infants (n=10)	1.36 ± 0.10	1.35 ± 0.13	1.38 ± 0.13	1.36 ± 0.12	1.35 ± 0.12
Mean number of the recovered infants	5.75 ± 1.35	5.66 ± 1.07	5.75 ± 0.75	5.50 ± 1.44	5.08 ± 1.16
*Mean weight of 50 day old offsprings (n=10)	24.53 ± 1.46	22.77 ± 2.02	12.01 ± 2.14	14.42 ± 2.43	12.66 ± 1.87

*The significant difference ($P < 0.001$) between the weight of 50 day old offsprings in the control and treatments is >50 mg/kg (one-way ANOVA test) §. The numbers given in round bracket indicates day after first gestation @ mean weight of the pregnant mice, mean weight, mean length of gestation, mean weight and number of infants did not show significant difference between all of the groups (One-way ANOVA test).

Table 2. Mean number of germ cells in the 60-70 day old offsprings in the treatment group (except dose 50 mg/kg) and control group.

Parameter	Control	Treatment
Mean spermatogonium	43.20 ± 10.52	21.25 ± 7.97*
Mean spermatocyte I	46.50 ± 9.82	31.35 ± 5.87*
Mean spermatid	140.15 ± 22.22	76.85 ± 28.95*

*The significant different ($P< 0.001$) in the control and treatments is >50 mg/kg (One-way ANOVA). The 60 to 70 day old offsprings who had slow and abnormal growth (higher than 50 mg/kg group).

DMSA-coated Fe_3O_4 and size of particle relative to DMSA-coated as additional shell controllability and magnetic properties for ferrite with and without DMSA. Also, magnetic saturation (M_S) have ratio 32:90 emu/g for DMSA-coated Fe_3O_4 / Fe_3O_4, indicating that, nonmagnetic DMSA molecules were bounded on the surface of Fe_3O_4 and reduced magnetization of Fe_3O_4. These findings are consistent with the previous reports (Chen et al., 2008; Shen et al., 2007; Lee et al., 1996).

As can be seen in Table 1, injection of iron oxide nanoparticles coated with DMSA at doses of 50, 100, 200 and 300 mg/kg had no adverse effect on weight changes of adult mice even after three months. These observations supported the data reported by Hafeli and Pauer, 1999; Kim et al., 2006, using different nanoparticles. Although, the doses higher than 50 mg/kg apparently had no effect on gestation and fetal growth, these doses led to a significant decrease in the infant's growth and maturation after birth and caused about 70% death before reaching puberty (Table 1). The histological examinations showed that, DMSA-coated nanoparticles exist in the placental tissue (Figure 4). Also, the accumulated iron oxide nanoparticles were detected in the sinusoids and hepatocytes of the fetus liver (Figure 5). Therefore, the DMSA-coated Fe_3O_4 nanoparticles were passed via blood-placenta barrier and distributed to the fetus liver and most likely undeveloped or under developed organs. This is in accord with the results reported by other investigators, in which injection of nanoparticles to mice resulted in the presence of the iron oxide particles in the brain (Kuckelhaus et al., 2003), testes (Kim et al., 2006), lung (Garcia et al., 2005) and offsprings testes of prenatal exposure to nano-sized TiO_2 (Takeda et al., 2009), which indicated the cross of nanoparticles via blood barriers.

The histological study of testes tissue sections indicated significant reduction on the number of spermatogonia, spermatocytes I, spermatids and mature sperms in seminiferous tubules of male offsprings (Table 2 and Figure 6). Indeed, in the same study, was reported the side effect of TiO_2 nanoparticles on the genital system development of mice offsprings together with disruption and disorganization in seminiferous tubules and led to decreasing of mature sperms (Takeda et al., 2009). However, further studies are required to examine the fate of the female pups following the nanoparticle toxification.

The results of the present study indicate that, although iron oxide nanoparticles had little side effects on adult animals, the presence of the particles in the placenta and fetus might disrupt the critical period of the fetal organ as growth and development, such as testes. Therefore, we concluded that the widespread use of iron oxide nanoparticles by pregnant mothers might have severe side effects on their infants after birth.

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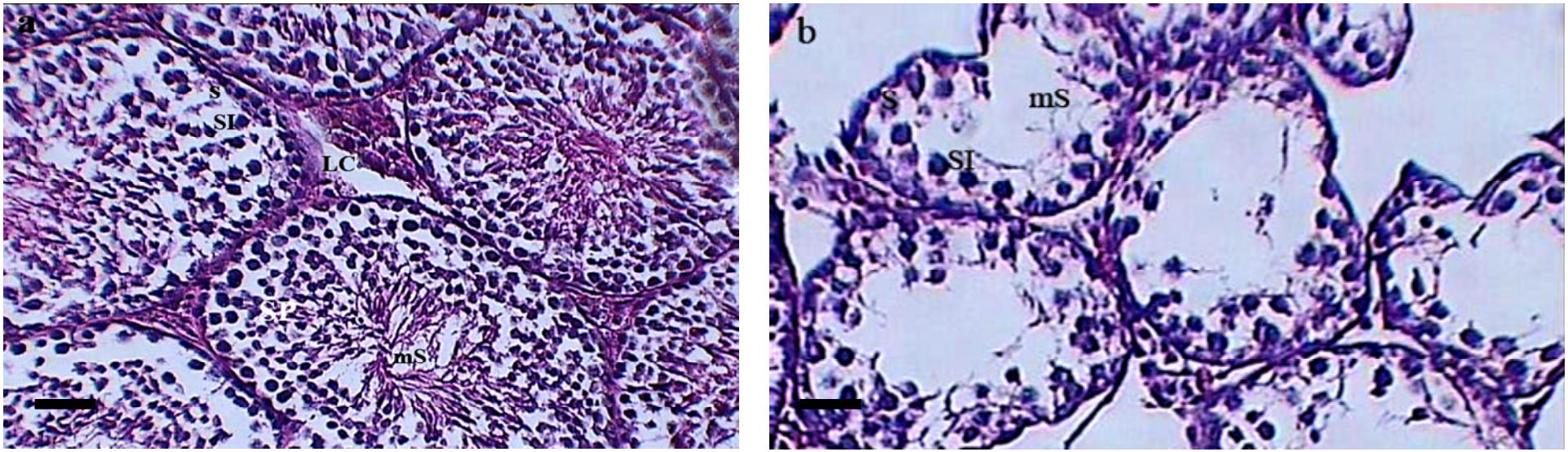

Figure 6. The testis tissue sections (hematoxilin-eosin, ×400) of 60 to 70 day old infants. (A) Control group; (b) treated groups more than 50mg/kg (S, Spermatogonium; SI, spermatocyte I; SP, spermatid; Ms, mature sperm; Lc, Lydig cells).

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