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The effect of aerobic exercise on hepatotoxicity induced by intratracheal instillation of iron oxide nanoparticles in Wistar rats

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Abstract. Iron oxide nanoparticles ($IONP_S$) can cause significant health problems due to their unique physicochemical properties and environmental characteristics. They are found as ultrafine particles in ambient air. After inhalation, these particles move from the lung to phagocytosis tissues, especially the liver. The aim of present study was to investigate the effect of concurrent aerobic exercise and $IONP_S$ on liver enzymes and histological hepatic appearance. 48 rats were divided into six groups: experimental 1 (aerobic exercise), experimental 2 (nanoparticle, anesthesia), experimental 3 (aerobic exercise, nanoparticles, anesthesia), placebo 4 (distilled water, anesthesia), placebo 5 (aerobic exercise, anesthesia), and control group. In groups 2 and 3, 40 mg/kg/b.w. of $IONP_S$ was injected *via* intratracheal installation every other day for 14 days. Groups 1, 3, and 4 run on treadmill for 30 minutes with the intensity of 35–40% VO2max (maximal oxygen consumption) every day. ALT was increased in group 1 but decreased in groups 2 and 3. AST was not significant in any of the groups, while ALP was reduced significantly in groups 2 and 3 ($p < 0.05$). Histological examination of the liver showed that, in groups 2 and 3, hepatic cells were damaged and also the congestion, inflammation, mononuclear cell infiltration, and ballooning degeneration were occurred. Tissue injuries in group 3 were less than those of group 2. These findings indicated that hepatotoxicity was caused by iron oxide nanoparticles; however, low-intensity aerobic exercise could decrease the damage somewhat.

Key words: Aerobic exercise — Iron oxide nanoparticles — Hepatotoxicity — Liver enzyme — Ultrafine particles

Introduction

Special physicochemical characteristics of nanoparticles (NPs), including size, high specific surface area, reactivity, and shape, facilitate the entry of these substances into the organisms' bodies and their movement into tissues, cells, although it may not be possible for larger particles to enter (Buzea et al. 2007; Kovochich et al. 2007). Iron oxide nanoparticles (IONPs) naturally are found as ultrafine particles in ambient air (Huang et al. 2006). Magnetites or maghemites are the most common types of IONPs that are produced from traffic, industry, and power stations (Huang et al. 2006; Apopa et al. 2009; Indira and Lakshmi 2010). NPs that spread in the environment through natural ways or engineered processes may be suspended in a gas as a nanoaerosol and enter the body through respiratory pathways (Terzano et al. 2010; Ferreira et al. 2013). Despite the importance of the respiratory effects of NPs, only a few studies have examined these effects (Zhu et al. 2009; Park et al. 2010). Precipitated NPs can overcome pulmonary tissue barriers and move out of the respiratory system. NPs pass through the respiratory epithelium, enter the circulatory system, and spread throughout the body (Wallenborn et

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al. 2007; Liang et al. 2008; Szalasy et al. 2011). Heart, liver, and kidney tissues are affected by nanoparticles (Sadauskas et al. 2007; Ai et al. 2011). IONPs enter *via* endocytosis into Kupffer cells, sinusoids and macrophages in the spleen (mono nuclear phagocyte system, MPS) (Gu et al. 2012). IONPs can lead to an increase in the permeability of endothelial cells in human macrophages through ROS (reactive oxygen species) generation which subsequently can cause inflammation (Revell 2006; Apopa et al. 2009). These particles are then integrated into the hepatocytes by the bloodstream and cause reduction in mitochondrial activity and morphological changes in hepatic cells (Revell 2006; Apopa et al. 2009). Also, they induce oxidative stress response in hepatocytes by generating free radicals and cause hepatic cell death (Sanvicens and Marco 2008). In addition, IONPs' reaction with proteins and enzymes in hepatocytes cause structural changes and will ultimately cause the liver cells to die (Hodges et al. 1995). Changes in biochemical parameters including serum bilirubin (TBIL), alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST) along with other hepatic pathological factors can cause peripheral and central venous disorders as well as patchy necrosis of the liver which provide ways and means for the evaluation of NPSs' effects on liver function (Liang et al. 2008). Because of the vital role of the liver in maintaining homeostasis and energy supply, it is necessary to take these organs (heart, liver, and kidney) into consideration when we evaluate the changes resulting from exercise and physical activity (Johnson et al. 2009; Hallsworth et al. 2011). During exercise, several events occur that can help skeletal muscles to use energy and accelerate the metabolism of glucose and fatty acids, increasing fat oxidation and reducing blood triglycerides and cholesterol and subsequently increasing the serum HDL concentration. Also, during physical activity some mechanisms are activated that can affect hepatic cells (St. George et al. 2009; Marques et al. 2010). Therefore, regular exercise causes changes such as gene expression in hepatocytes, protection from hyperglycemia, and hepatic steatosis. Additionally, it improves some metabolic parameters in patients with fatty liver (Lee et al. 2006; Gutierrez-Grobe et al. 2009). Different intensities of exercise have impacted the liver greatly and lead to some other effects (Yi et al. 2013). Regular exercise causes reduction in ROS through anti-oxidation system compatibility (Ploeger et al. 2009; Sun et al. 2010). Exercise can temporarily increase liver function tests, but researches still must conducted in order to reveal how exercise leads to an increase in the amount of biochemical factors (Pettersson et al. 2008). Despite the importance of examining the effects of physical activity on the absorption of inhaled NPs and their effects on the liver disorders, no study has been done in this field. Also, the respiratory effects of the nanoparticles commonly used in industry have not been fully studied; hence their potential for hazardous effects on human health still remains unclear (Park et al. 2010a). Therefore, this study aimed to determine the effect of low-intensity aerobic exercise on hepatotoxicity induced by intratracheal instillation of IONPs.

Materials and Methods

Iron oxide nanoparticles; γ-Fe_2O_3

IONPs were obtained from US Research Nanomaterials, Inc. (Houston, USA). XRD (X-Ray Diffraction) results of nano γ-Fe_2O_3 are in crystalline phase with 20 nm size. Purification of nano γ-Fe_2O_3 was determined as 99.5% by using ICP-MS. Table 1 summarizes the characteristics of nano γ-Fe_2O_3 used in the present study. XRD pattern and the size distribution of the nano γ-Fe_2O_3 particles are also shown in Fig. 1 and Fig. 2.

Animals

Forty-eight male Wistar rats were obtained from the Pasteur Institute in Tehran, Iran. The animal studies were performed in accordance with the protocols of care and use of experimental animals. This project was approved by the ethics committee of the Medical University of Isfahan, Iran and was recorded in the clinical trial center with registration number IRCT2014021816624N1. Laboratory animals were kept on a 12-h light/dark cycle at a temperature of 22–24°C and a relative humidity of 50 ± 5%. The rats were protected from possible stress and were fed a standard diet of commercial rat chow. Adult male Wistar rats with an average weight of 250–300 g were randomly divided into 6 groups (8 in each group) as follows: experimental group 1: run on

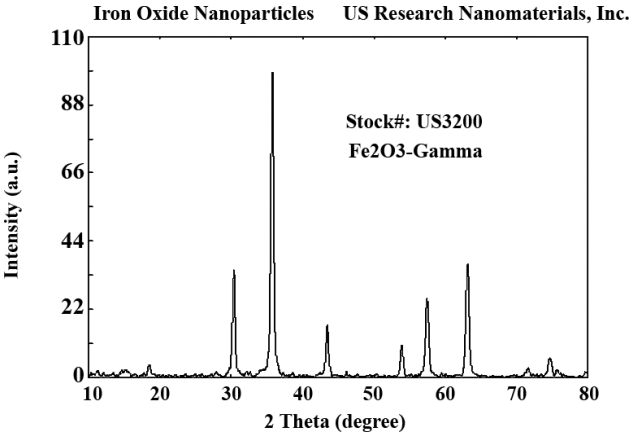

Figure 1. XRD patterns of γ -Fe_2O_3 nanoparticles.

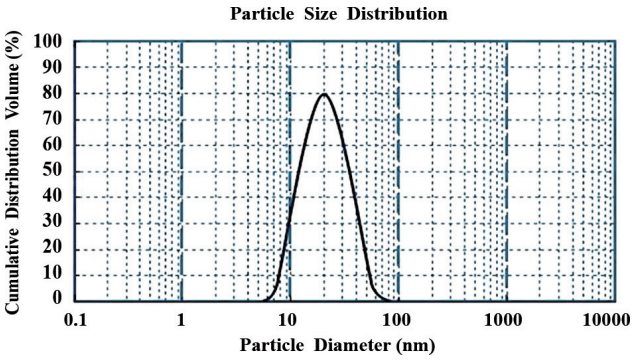

Figure 2. Size distribution of nano γ-Fe_2O_3.

the treadmill; experimental group 2: injected with 40 mg/kg/b.w. of IONPs; experimental group 3: injected with 40 mg/kg/b.w. of IONPs seven times and run on the treadmill; placebo group 4: injected with distilled water; placebo group 5: received both anesthesia and running on treadmill; control group. In this study, the treadmill was used with an intensity of 35–40% VO2max during 14 days of study. The injection of IONPs or distilled water was performed seven times (every other day) and each time the rats were anesthetized with ether. The control group had the same environmental experimental conditions but without the exercise, injection or anesthesia.

Intratracheal instillation method

In this study, the intratracheal instillation method was used to examine the toxic effects of inhaled NPs. The advantage of this method is the receiving of the full injected dose in the host's respiratory tract (Oursgaard 2009; Park et al. 2010b; Nalabotu et al. 2011). In intratracheal instillation, for every injection, 0.1 ml of 100 mg/ml of IONPs in distilled water was injected into the rat's trachea. The required dosage was 40 mg/kg/bw. Administration was repeated seven times every other day during a period of 14 days. At each injection, the rats were anesthetized by inhalation of ether and weighed.

Training protocol

Male Wistar rats were trained to do low-intensity aerobic exercise in accordance with the protocol used by Howely et al. (1995). Rats were run on a motor-driven treadmill designed for rats with an intensity of approximately 35–40% VO2max (MTM-5720, Proteus, Taiwan) for 30 minutes each day for 2 weeks. The exercise sequence was as follows: exercise for 5 min at a speed of 6 m/min, exercise for 5 min at a speed of 9 m/min and exercise for 20 min at a speed of 12 m/min with a running intensity of 35–40% VO2max. To eliminate the exercise machine as a factor, both placebo groups and the experimental group 2 were placed on a nonoperational treadmill for 30 min *per* day for two weeks. In order to reduce stress in the animals and to adapt them with the conditions of the exercise, the running groups had a pre-adaptation period of one week in which they ran 15 min a day on a treadmill at a speed of 9 m/min.

Histopathology examination and enzymology method

Twenty-four hours after the last exercise session, the animals were sacrificed according to animal protection regulations, their blood samples were centrifuged at 3000 rpm for 15 minutes and the serum was introduced to biochemical auto analyzer (Hitachi-717, Germany-Japan) to measure the amount of enzymes (ALT, AST, ALP). Liver enzymes were measured by using enzymatic kits (Greiner, Germany) with IFCC method. For histopathology examination, the liver was removed and the tissue sections were fixed in 10% formalin for hematoxylin-eosinophil staining. Hepatic tissue sections were examined by light microscope.

Statistical analyses

Statistical analyses were performed with SPSS software. A one-way Analysis of Variance (ANOVA) was used to evaluate the statistical significance. *t*-test and Duncan test were applied and the level of significance was set at $p < 0.05$.

Results

Evaluation of serum levels of ALT, AST and ALP

The results of the serum levels of ALT, AST and ALP are presented in Table 2 and the results of the Duncan test for ALT and ALP analysis are shown in Tables 3 and 4, respectively. In the experimental group 1 the exercise increased the levels of ALT, while in the experimental groups 2 and 3 the injection of pulmonary IONPs reduced the ALT levels to a meaningful degree. Furthermore, the results of the Duncan test indicated that in experimental group 3, IONPs

Table1. Physical parameters of nano γ-Fe_2O_3 used in present study

Color	brown
Morphology	spherical
Crystalline phase	gamma
Specific surface area	40–80 m^2/g
Size	20 nm
Purity	99.5%

Table 2. Group comparison study of serum enzymes ALT, AST and ALP

Group	ALT (u/l)	AST (u/l)	ALP (u/l)
Experimental group 1 (aerobic exercise)	64.12 ± 10.49*	147.12 ± 22.8	561.50 ± 79.02
Experimental group 2 (nanoparticle, anesthesia)	30.00 ± 4.89**	209 ± 205.65	269.62 ± 130.20#
Experimental group 3 (aerobic exercise, nanoparticles, anesthesia)	40.5 ± 18.40##	186.37 ± 114.95	364.37 ± 105.31&
Placebo 4 (distilled water, anesthesia)	53 ± 16.37	198.50 ± 129.21	578.37 ± 76.67
Placebo 5 (aerobic exercise, anesthesia)	67.25 ± 10.08	313.37 ± 247.11	578 65 ± 76.50
Control group	48 ± 5.31	133.12 ± 10.98	597.25 ± 144.71

* $p < 0.0001$ *vs.* Control (t-test); ** $p < 0.001$ *vs.* Control and Placebo 5 (F = 10.695; ANOVA); # $p < 0.001$ *vs.* Control and Placebo 5 (F = 18.55; ANOVA); ## $p < 0.0001$ comparisons between six groups (F = 11.01; ANOVA); & $p < 0.0001$ comparisons between six groups (F = 13.84; ANOVA).

reduced ALT independently, but the effect of exercise intervention depressed the effect of NPs on enzyme ALT, and experimental group 3 ranked slightly better than experimental group 2. These results indicated that the training intensity in group 3 was affected by inhalation of IONPs during the two week period ($p < 0.05$). Evaluation of serum levels of AST showed no significant differences among different groups ($p > 0.05$). No significant difference was seen for the serum levels of ALP in experimental group 1, while the serum levels of ALP decreased in experimental groups 2 and 3 ($p < 0.05$).

Histopathological evaluation

Microscope slides were prepared from liver tissue showed that there was no change in hepatic cells of experimental group 1 compared with those of control group (Fig. 3a). In the experimental groups 2 and 3, inflammation and mononuclear cell infiltration in centrilobular hepatocytes were observed (Fig. 3b,c), also the increased inflammation and spotty mononuclear cell infiltration in liver parenchyma that underlie necrotic spots were observed. There was no difference in the severity of injury between these two groups (Fig. 3d,e). In the experimental group 2 and experimental group 3, centrilobular venous congestion and centrilobular sinusoid congestion was observed, but the intensity of congestion in the experimental group 2 was more than that of the experimental group 3 (Fig. 3f,g). In the experimental group 2, ballooning degeneration in priportal hepatocytes was observed, while in the experimental group 3 this disorder was not seen (Fig. 3h). The structure of hepatic cells in group 3 was healthier and had fewer tissue abnormalities than those of experimental group 2, and macrophage cell density was reduced in this group, so the severity of the lesions was less than that observed in experimental group 2.

Discussion

Most studies in nanotoxicology have emphasized the fact that NPs, based on their chemical composition, shape, size, surface chemistry, surface charge and specific surface area cause different effects and responses in different parts of the organism's body (Auffan et al. 2006). Iron oxide nanoparticles have potential of mobility from the lungs to other organs after inhalation. Once moved, they interred into the blood stream from pulmonary artery and they trapped by the reticuloendothelial system, which is part of the immune system. Therefore, liver, spleen and kidneys are the

Table 3. Duncan test to investigate enzyme ALT

Group	N (sample size)	Subset for alpha = 0.05			
		1	2	3	4
Experimental group 2 (nanoparticle, anesthesia)	8	30.0000			
Experimental group 3 (aerobic exercise, nanoparticles, anesthesia)	8	40.5000	40.5000		
Placebo 4 (distilled water, anesthesia)	8		48.0000		
Experimental group 2 (nanoparticle, anesthesia)	8		53.0000	53.0000	
Experimental group 1 (aerobic exercise)	8			64.125	64.125
Placebo 5 (aerobic exercise, anesthesia)	8				67.2500
Significant level		0.089	0.055	0.072	0.607

target organs for these particles, so an increasing number of Fe_2O_3 nanoparticles in macrophage environments may be considered as an overload for these cells and they are unable to filter these particles to desirable levels (Zhu et al. 2009). In many liver diseases, the levels of liver enzymes are measured (Narendhirakannan et al. 2007). The results of this study showed that aerobic exercise could not prevent the decrease in serum levels of ALT and ALP in the experimental group 3. However, the statistical analysis indicated that the exercise led to the greatest changes in the serum

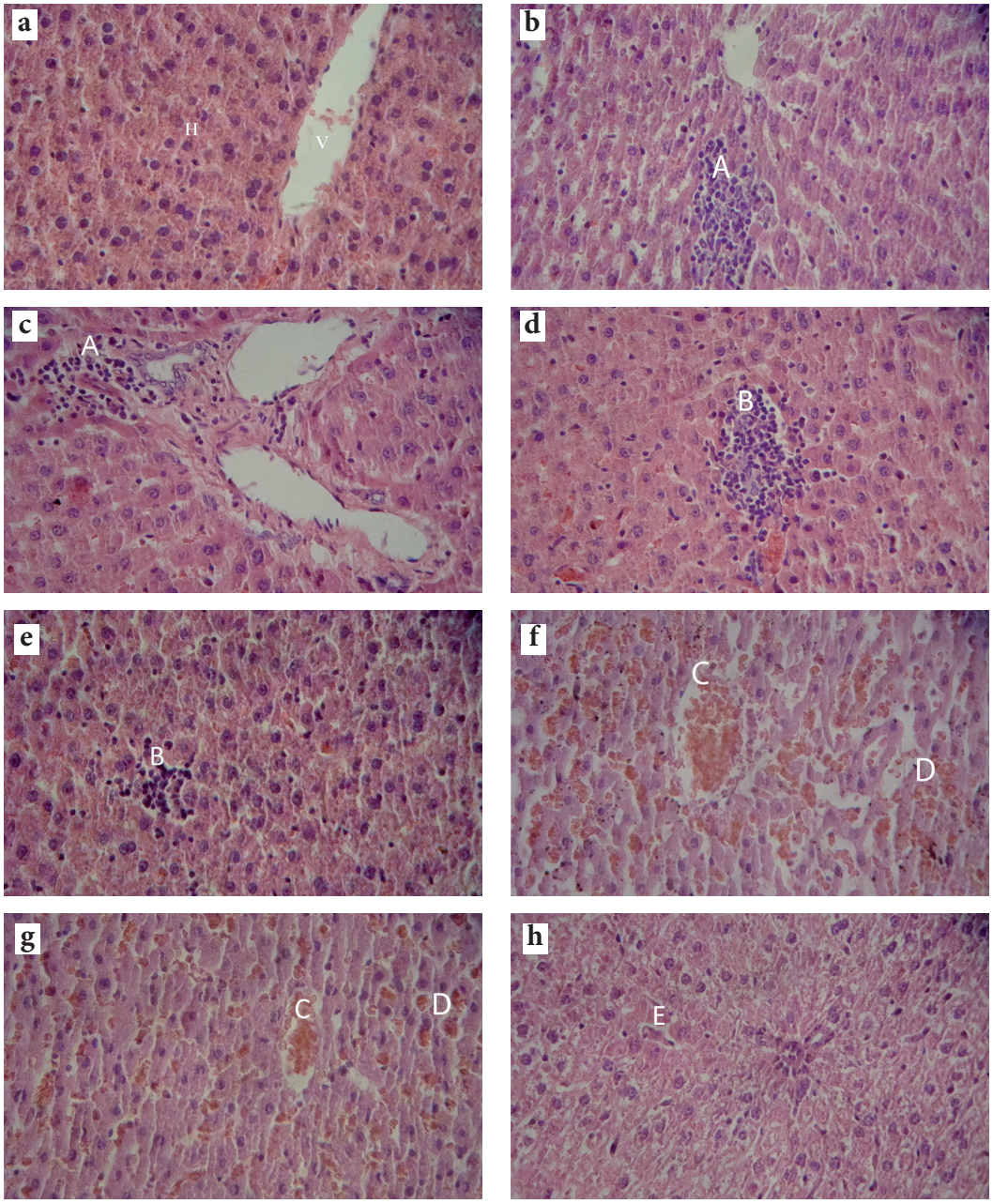

Figure 3. Histopathological evaluation of the liver tissue in different groups. **a.** Control group. Cells stained with hematoxylin-eosin showed normal structure. Mononuclear cell infiltration in centrilobular hepatocytes (A) in experimental group 2 (**b**) and experimental group 3 (**c**). There was no difference in the severity of injury between these two groups. Spotty mononuclear cell infiltration in liver parenchyma (B) in experimental group 2 (**d**) and experimental group 3 (**e**). Centribular venous (C) and sinusoids (D) congestions are more severe in group 2 (**f**) than in group 3 (**g**). Ballooning degeneration in priportal hepatocyte (E) in experimental group 2 (**h**) was clearly visible. V, lobular central vein; H, hepatocytes. Magnification ×400; Hematoxylin and eosin staining.

level of ALT enzyme, as the NPs had a suppressive effect on this enzyme whereas the training had an increasing effect on it. However, with approaching the group 3 to the control in term of serum levels of this enzyme, the exercise treatment modulated the effect of the NPs on the serum levels of this enzyme.

Studies have shown that the exercise can affect the peroxidation of fatty liver cells, cause overload on the liver cells or hypoxic liver cells, and reduce energy supplies to increase the amount of ALT enzyme (Fojt et al. 1976; Sreenivasa Baba et al. 2006; Bürger-Mendonça et al. 2008). In addition, ALP is a marker enzyme for the plasma membrane of endoplasmic reticulum system (Tokumitsu and Fishman 1983). Damaged membrane cells may affect ALP activity and NPs have also the potential to disable ALP. Especially low levels of ALP in serum are also an indicator for liver dysfunction (Wang et al. 2010). On the other hand, ALP enzyme is not produced by the muscles (Suzuki et al. 2006; Pettersson et al. 2008; Gutierrez-Grobe et al. 2009). In addition, stress in hepatocytes, followed by aerobic exercise of low intensity, cannot change the values of these enzymes originating in the liver. Hence, the reduction in the amount of the serum levels of this enzyme in the experimental groups 3 and 2 are in line with each other. The results of the present study did not show any significant changes in serum AST levels in the experimental group 2 which therefore showed no severe damage to the hepatocytes and non-stimulated mitochondrial liver tissues. Due to the more severe damage in hepatocytes and damage to mitochondria, the release of AST and the ratio of AST/ALT would have expected to increase (Ramaiah 2007). In fact, a large increase in the levels of mitochondrial AST can cause a massive increase in liver necrosis (Thapa and Walia 2007), but in this study the necrosis of liver tissue was not seen. Since the AST exists in liver cells, heart, muscle, leukocytes and erythrocytes (Kinoshita et al. 2003), no significant changes in the experimental group 1 and experimental group 3 in this study, indicated that the exercise may have no effect on these cells. Previous researches proved the movement of IONPs from the lungs to the liver, their accumulation and causing damage to liver tissue (Pardoe et al. 2003; Chaves et al. 2005; Garcia et al. 2005; Wang et al. 2010; Noori et al. 2011; Xin-Li et al. 2012; Prodan et al. 2014). This is of special important because the liver is the first organ which due to biotransformation of toxins after intratracheal instillation is exposed to the adverse effects of nanoparticles (Noori et al. 2011; Wang et al. 2010; Prodan et al. 2014). In terms of histology, in the current study, experimental groups 2 and 3 showed tissue disorders compared with the control group. Some of these disorders are including mononuclear cell infiltration in centrilobular hepatocytes, spotty mononuclear cell infiltration in liver parenchyma, centrilobular venous congestion, centrilobular sinusoid congestion, and ballooning degeneration in priportal hepatocytes.

Researchers claimed that the increased permeability and accumulation of IONPs in tissues, pro-inflammatory factors, and macrophage oxidative stress are the main reasons for changes in liver tissue (Guo et al. 2009; Priprem et al. 2010; Wang et al. 2010). In this study, the extent of damage to the liver is reduced in the experimental group 3, indicating that aerobic exercise can reduce the adverse effect of NPs. One of the possible reasons is that after exercise, we see nanoparticle effects on liver disorders, hepatic blood flow and reduced contact of NPs with the cells of the liver. Exercise reduces blood flow to the visceral area, thus reducing the body's absorption of drugs in the gastrointestinal system (Peng and Cheung 2009). Exercise also affects the amount of available medicine cell receptors which have a pronounced effect on the activity of drug kinetics (Somani et al. 1990). Another mechanism that is a common topic in exercise physiology and toxicity of nanoparticles is the mechanism of oxidative stress on the cell surface and its increasing by NPs and its reduction due to aerobic exercise. In fact, aerobic exercise reduces the effect of nanoparticles in the experimental group 3 due to reduction in the level of oxidative stress.

Studies have shown that the most important factor-induced hepatic toxicity due to NPs is oxidative stress (So-

Table 4. Duncan test to investigate enzyme ALP

Group	N (sample size)	Subset for alpha = 0.05	
		1	2
Experimental group 2 (nanoparticle, anesthesia)	8	269.6250	
Experimental group 3 (aerobic exercise, nanoparticles, anesthesia)	8	364.3750	
Placebo 4 (distilled water, anesthesia)	8		561.5000
Experimental group 2 (nanoparticle, anesthesia)	8		578.2500
Experimental group 1 (aerobic exercise)	8		578.3750
Placebo 5 (aerobic exercise, anesthesia)	8		597.2500
Significant level		0.80	0.544

mani et al. 1990; Fu et al. 2014). After 12 hours of induction of IONPs, an increase in the rate of superoxide dismutase (SOD) and decline in the glutathione peroxidase (GSH) levels in cells were observed (Wang et al. 2009). This is in contrast to how gentle and regular exercise contributes to strengthen the ability of antioxidants to reduce oxidative damage and increase resistance to oxidative stress (Falone et al. 2010). Regular exercise increases antioxidant enzymes, DNA repair proteins, and mitochondrial electron carrier proteins that regulate the production of ROS (Marques et al. 2010). Also, after both endurance and aerobic exercise an increase in mitochondrial ROS hepatocytes has not been seen due to an increase in antioxidant defenses, such as increased levels of GSH and NQO-1 enzyme (Lannig et al. 2005). In fact short-term aerobic exercise can delay serum apoptosis factors by decreasing oxidative stress factors and increasing anti-oxidant defenses (Ogonovsky et al. 2005; Fealy et al. 2012).

Submaximal exercises also strengthen cytochrome P450 as a crucial factor in removing xenobiotic factors from the liver (Frenkl et al. 1980) so as to increase the capacity of the cytochrome P450 enzymes in rats through increased mRNA synthesis (Dyke et al. 1998). The low-intensity aerobic exercise (35–40% VO2max) used in the present study increased the antioxidant system in experimental group 3, so the amount of tissue abnormalities compared to experimental group 2 is reduced.

In conclusion, our findings suggest that low-intensity aerobic exercise (35–40% VO2max) over a period of 14 days significantly affected hepatotoxicity induced by intratracheal instillation (7 times injected 40 mg/kg/bw) and somewhat reduced toxic effects of IONPs. However, since aerobic exercise induces many physiological processes in the body, evaluation of the interaction between aerobic exercise and nanoparticles' effects on organisms' function needs further study.

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