Original Article

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Comparison of the effects of zinc oxide and zinc oxide nanoparticles on the expression of hepcidin gene in rat liver

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Abstract

Objectives: Nanoparticles have special properties, such as increased intestinal absorption, permeability, and so on. Zinc oxide (ZnO) nanoparticles have medical applications such as using in drug production. Studies of ZnO nanoparticles have shown the role of these particles in reducing or increasing the genes expression. Given the important role of hepcidin in the development of anemia and iron overload diseases, this study investigated the effect of ZnO nanoparticles on the hepatic expression of the hepcidin gene to help find a way to treat these diseases.
Methods: In this experimental study, 24 male Westar rats were divided into three groups: control, ZnO treating group and ZnO nanoparticle treating group. Both ZnO and ZnO nanoparticles were injected with 50 mg/kg body weight for 14 days. At the end, serums were collected and iron, ferritin and IL-6 levels were measured. Expression of the hepcidin gene was done by Real Time PCR.
Results: ZnO and the ZnO nanoparticle significantly increased the expression of the hepcidin gene relative to the control group. The increase in expression of the hepcidin gene in ZnO nanoparticles was more significant than in the ZnO.
Conclusion: ZnO nanoparticles led to significant increase in expression of the hepcidin gene.

Keywords: hepcidin; ZnO; ZnO nanoparticles; Wistar rat.

Introduction

There are several number of elements in nature that perform vital functions in human body and are essential for the growth and survival of life [1, 2]. One of these important elements in the human body is iron, which plays an important role in many reactions in the body. Iron hemostasis occurs only through changes in its absorption level, and any defects in the metabolic pathway of iron can lead to an increase in the body's iron or anemia [3, 4]. Regulation of the body's iron level requires mechanisms which control the absorption and its release from its reserves. Therefore, the relationship between the cells that consume iron and the cells that absorb iron must be precisely regulated. One of the important proteins for regulating iron hemostasis is hepcidin, a small cysteine-rich peptide [5–8]. Hepcidin mainly secreted by liver cells, but also, it has been synthesized by other tissues such as the brain, heart, kidneys, retina, monocytes, macrophages, spleen, lungs, adipose tissue cells, and pancreatic beta cells. Some studies have shown that a decrease in the expression of the hepcidin gene or a lack of synthesis causes a significant increase in iron load, and conversely, its high expression causes chronic anemia [9]. Studies have shown that one of the causes of acute liver failure can be high iron intake, which is often seen in children under six years of age and pregnant women [10]. Frequent and regular blood

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transfusions in patients with blood disorders such as thalassemia, myelodysplastic syndromes, and aplastic anemia cause secondary iron overload. Excess iron can cause destructive damage to the gastrointestinal tract and lead to stomach ulcers, bleeding and shock. Cellular mechanism involve in iron overload toxicity, including formation of free radicals such as radical hydroxyl during the reaction of fenton and so on [11]. Therefore, any factor that causes a change in the increase or decrease in the expression of the hepcidin gene may cause an overload of iron or anemia by increasing or decreasing the levels of iron in the body. In recent years, nanotechnology has advanced rapidly [12]. Nanoparticles have unique properties such as penetration into cells much more easily due to the small size of the molecule and also increase the penetration [13–16]. Among nanoparticles, zinc oxide (ZnO) nanoparticles are widely used in medicine [17, 18]. Some researchers have been considered ZnO nanoparticles as low-toxicity materials. Because zinc is an essential trace element in the human body, it is added to foods as a dietary supplement. Studies on ZnO indicate the role of these particles in increasing or decreasing the expression of some genes that can play an important role in reproductive processes and cell division, and etc. [19]. Due to the important role of hepcidin protein in the development of anemia or its role in iron overload diseases, this study aimed to investigate the effect of ZnO and ZnO nanoparticles on the hepatic expression of hepcidin gene and compare their effects with each other to find a way to cure diseases related to iron metabolism.

Methods and materials

Reagents

Iron measurement Kit (Darman-kave), ferritin measurement Kit (Abcam, Cat No: Ab157732) IL-6 measurement (Ebioscience, Cat No: BMS625) csDNA Synthesis Kit (Fermentase), PCR Kit, TBE buffers, ladder, loading dye, DNA safe stain (Sina Clone), Real Time Kit (EURex), ZnO, and ZnO nanoparticles (CAS NO1314-13-2, Molar Mas: 81.39 g/mol, Size: <100 nm, sigma) were purchased.

Animal study and sampling

In this study, 24 adult Wistar rats with an average weight (180 ± 20 g), were purchased from the laboratory animals' department of Tehran University of Veterinary Medicine. The animals were kept at a temperature of 22.2 °C, with a cycle of 12 h of light and 12 h of darkness. They were then randomly divided into three groups (control, ZnO treatment group and ZnO nanoparticles treatment group). Control group, received only 1 cc of physiological serum intraperitoneally for 14 days. The second group (ZnO treatment group) received 1 cc of physiological serum containing 50 mg/kg ZnO as an intraperitoneal injection for 14 days. The third group (ZnO nanoparticles treatment group) received 1 cc of physiological serum containing 50 mg/kg ZnO nanoparticles by intraperitoneal injection for 14 days. Twelve hours after the last injection, the rats were first anesthetized with ether, and after opening the abdominal cavity and chest, blood was drawn directly from the heart. The liver was then removed immediately and transferred to a sterile plate after rinsing in saline. 0.5 g was isolated from liver tissue and crushed by scalpel and transferred to 1.5 mL vials. The vials were first labeled and then transferred to a laboratory inside a container containing liquid nitrogen. Animal blood was centrifuged at 2000 rpm for 20 min and measured iron, ferritin, and interleukin-6 levels using commercial kits in the serum. This research has been approved by the Ethics Committee of Ardabil University of Medical Sciences (ID: IR.ARUMS.REC.1395.132) in terms of animal study ethical standards.

Real time PCR

Total RNA of the liver tissue sample was extracted by Trizol according to the manufacturer's protocol. Concentration of total amount of RNA was determined by optical density measurement (A260/A280 ratio) by Nano Drop (Wilmington, DE, USA). cDNA was synthesized by employing the Two-Strand Synthesis kit according to the manufacturer's protocols. RT-PCR was performed using SYBR Green-based PCR Master Mix and the gene expression was evaluated on a MIC system (Australia). Specific primers for investigating gene have been used (GAPDH, F: GTTACCAGGGCT, R: GGGTTTCCCGTT; Hepcidin, F: AAGATGGCACTA, R: GCATTTACAGCA). All samples were evaluated in triplicates. Quantitative interpretation of the data was carried out using ΔΔCt method with efficiency correction according to Pfaffl technique, and the CT (Cycle threshold) values were standardized with respect to GAPDH expression.

Statistical analysis

The quantitative results were analyzed after determining the mean and standard difference of the data in three independent experiments using SPSS software version 16

with One-way ANOVA test. P<0.05 values were considered statistically significant.

Results

Effect ZnO and ZnO nanoparticles on expression of hepcidin

Real time PCR was used to quantify the expression of hepcidin gene expression in the control group compared to the experimental groups receiving ZnO nanoparticles and ZnO. The results were shown in Figure 1. These findings showed that the expression of the hepcidin gene in ZnO nanoparticles and ZnO groups had a significant increase compared to the control group, however the increase in the ZnO nanoparticles treatment group was more significant.

Effect ZnO and ZnO nanoparticles serum iron levels

In this experiment, the serum levels of iron in ZnO and ZnO nanoparticles treatment group showed a significant decrease compared to the control group, however the reduction in the ZnO nanoparticles treatment group was more significant (Figure 2).

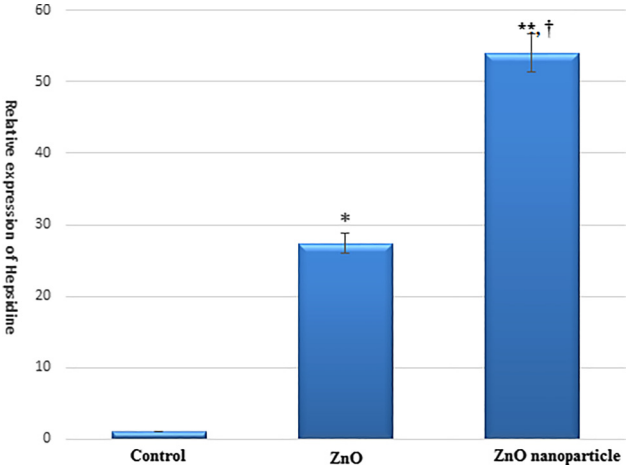

Figure 1: Comparison of hepcidin relative gene expression in the study groups.
* Indicates a significant difference relative to the control group (p<0.05). ** indicates significant significance difference compared to the control group (p<0.001). † indicates the significance difference of ZnO relative to the ZnO nanoparticle group (p<0.05).

Effect of ZnO and ZnO nanoparticles serum on ferritin levels

In this experiment, the serum levels of ferritin in ZnO and ZnO nanoparticles treatment groups showed a significant decrease compared to the control group, however the reduction in the ZnO nanoparticles treatment group was more significant (Figure 3).

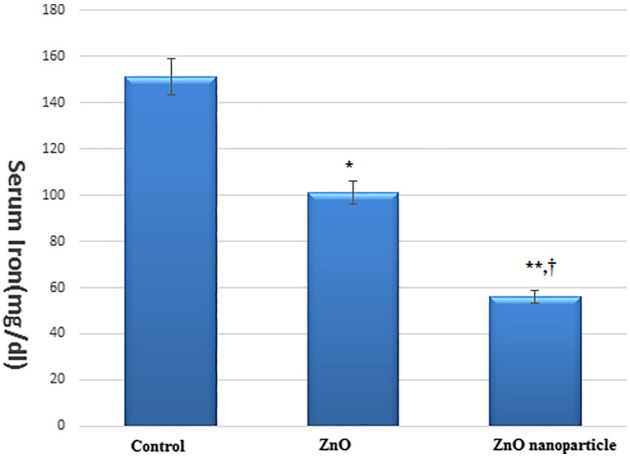

Figure 2: Mean of serum iron concentrations in the studied groups.
*Indicates a significant (p<0.05) relative to the control group.
**Indicates significant significance (p<0.001) compared to the normal control group. †Indicates the significance of (p<0.05) nanoparticles of zinc oxide relative to the zinc oxide group.

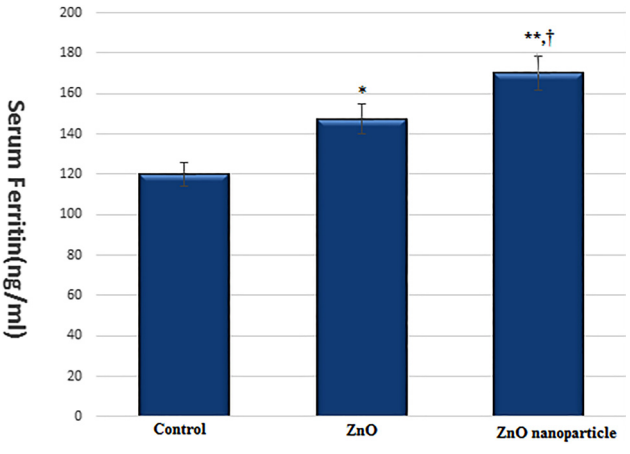

Figure 3: Mean of serum ferritin concentration in the studied groups.
* Indicates a significant difference relative to the control group (p<0.05). ** indicates significant significance difference compared to the control group (p<0.001). † indicates the significance difference of ZnO relative to the ZnO nanoparticle group (p<0.05).

Effect of ZnO and ZnO nanoparticles on serum IL-6 levels

In this experiment, serum IL-6 levels in ZnO and ZnO nanoparticles treatment groups showed a significant increase compared to the control group. The results also showed that there was a significant difference in serum IL-6 serum concentrations between ZnO and ZnO nanoparticles groups (Figure 4).

Discussion

Iron overload causes damage in liver, heart, pancreas, thyroid, and central nervous system, which are called iron overload syndrome. The main cause of damages due to iron overload in tissues, is the excessive production of oxygen free radicals (ROS). Iron overload either occurs genetically or secondary. The most common genetic defect of iron overload is hereditary hemochromatosis, which is more common in western countries, while forms of secondary hemochromatosis include types of induced by chronic hemolytic anemia, iron toxicity due to subcutaneous or muscular iron supplements, and major beta-thalassemia [20, 21]. Also, iron overload is caused by factors such as frequent blood transfusions (such as those seen in thalassemia patients). Therefore, the aim of this study is to achieve a way to deal with increase secondary hemochromatosis. In this regard, we investigate the effect of ZnO and ZnO nanoparticles on the expression of hepcidin gene. Before that, some studies reported the beneficial effect of ZnO nanoparticles reducing or increasing the expression of some genes in variety of disease [5, 7].

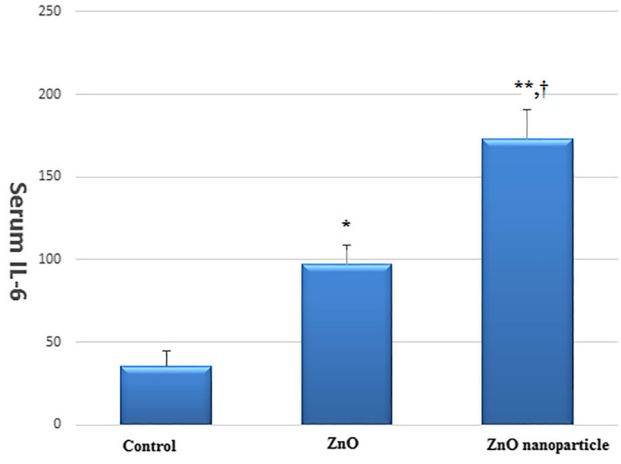

Figure 4: Mean of serum interleukin-6 concentrations in the studied groups.
*Indicates a significant difference relative to the control group ($p<0.05$). **indicates significant significance difference compared to the control group ($p<0.001$). †indicates the significance difference of ZnO relative to the ZnO nanoparticle group ($p<0.05$).

The results of this study showed that the expression of hepcidin gene in the ZnO and ZnO nanoparticles groups increased compared to the control group. Also, the expression of hepcidin gene in the nanoparticle group of ZnO is higher than ZnO group. On the other hand, according to the results of this study, in parallel with the increase in expression of hepcidin gene in the studied groups, serum iron levels decreased and this decrease is higher in the nanoparticle group of ZnO than ZnO. One of the toxic effects of ZnO nanoparticles and possibly ZnO, which has been considered by researchers in recent research, is the effect of these compounds on stimulating inflammatory responses and exacerbating disorders such as atherosclerosis. The study found that another effect of ZnO and ZnO nanoparticles was the increasing effect of these compounds on serum ferritin and IL-6 levels (as one of the main indicators of inflammation), which in both groups increased. Therefore, although serum iron levels decreased in this study, ferritin levels increased compared to the control group due to inflammation and increased acute phase proteins. Moreover, inflammation is a potent stimulant of hepcidin production [22, 23] which is along our study.

Hepcidin is a negative regulator of iron transport to the plasma that is produced by the liver and can interfere with iron metabolism in a variety of ways. Hepcidin can control the absorption of dietary iron by binding to deodorant enterocytes or by inhibiting the release of iron by binding to macrophages [24, 25]. In fact, hepcidin binds to ferroportin in the macrophage membrane of the anteroposterior region of the base and causes phosphorylation, induction of internal changes, stimulate the binding of ubiquitin to ferroportin, and finally decomposition of the ligand-receptor complex in lysosomes [5]. Removal of ferroportin from the cell membrane prevents the release of iron from the cell, leading to a reduction in the entry of iron into the plasma. Thus, the reaction between hepcidin and ferroportin can explain the systemic regulation of iron metabolism.

Hepcidin also reduces the absorption of iron in the intestines by reducing the levels of both Divalent metal transporter 1(DMT-1) and ferroportin, as well as reducing the release of iron recovered by macrophages from iron reserves, which results in a decrease in serum iron. Previous studies have shown that the elimination of the hepcidin gene causes severe forms of iron deficiency disease,

and in contrast, high hepcidin expression leads to decreased iron absorption and iron deficiency anemia [23].

Moreover, reduction in function of hepcidin, led to very severe forms of iron overload. In patients with secondary hemochromatosis, the excretion of iron stored in the tissues is considered an emergency because iron overload in these patients with Fenton reaction and exacerbation of oxidative stress and tissue destruction can lead to heart disorders. Vascular, liver cirrhosis or liver cancer, type 1 diabetes mellitus or chronic kidney disease may be involved. Also, the mortality rate in these patients is closely related to the amount of iron overload. Therefore, elimination of iron overload in these patients is one of the medical emergencies, and in this regard, various drugs have been evaluated, which have been limited due to severe drug side effects. On the other hand, we can use the body's natural mechanisms, such as increasing the expression of the hepcidin gene with much fewer side effects, to help remove excess iron stored in the body. One of the compounds that has recently attracted the attention of researchers in most fields of research, especially medicine, is the study of the medicinal properties of nanoparticles. It should be noted that limited studies have been performed on the effect of ZnO nanoparticles on the expression of some genes, including the study of Lee et al. that reported increase glutathione peroxidase and superoxide dismutase [26]. In a study by Fu J et al., which was performed on human bronchial cells, it has been showed that ZnO nanoparticles could increase the expression of the IL-8 gene [27]. As mentioned in the above studies, ZnO nanoparticles can have different effects on the expression of different genes, in some cases it has increased gene expression or in other cases it has reduced the expression of some genes. In this study, it was shown for the first time that the use of ZnO nanoparticles could increase the expression of the hepcidin gene. In this study, low doses of ZnO nanoparticles were used to minimize its toxic effects. In this regards, Wei-DongZhang et al. showed that ZnO nanoparticles stimulate side effects on organisms. So, protections should be taken when ZnO nanoparticles are used as diet additives because they might cause side effect and health issues [28].

The results of this study can be used in animal models of hemochromatosis to counteract the toxic effects of iron overload. It also appears that prolonged exposure to ZnO and ZnO nanoparticles could increase the expression of the hepcidin gene in people with iron-resistant anemia. Of course, our study in this area is still in its early stages, and further research is needed to determine the relevant mechanisms in this area.

Conclusion

The results of this study showed that injection of both dense and nanoparticles of zinc oxide increased the expression of hepcidin gene in the studied groups. The effect of zinc oxide nanoparticles on the expression of the hepcidin gene was more significant than the dense form of zinc oxide.

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Informed consent: Not applicable

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