Effect of subacute exposure of nano Zinc particles on oxidative stress parameters in rats

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Key words:
FRAP, GPx, nano Zinc, oxidative stress, rat

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Received: 24 October 2016
Accepted: 7 February 2016

Abstract:

BACKGROUND: Zinc (Zn) is one of the most important essential elements in the body of animals and plants. Zinc plays a significant role in the structure of more than 300 different proteins and in many life supporting biochemical and metabolic processes such as cellular respiration and protection against free radicals. Nanoparticles of zinc are the new form of Zinc used in cosmetic and personal care products and also in livestock feed and food packaging. **OBJECTIVES:** The aim of this study was to evaluate the effects of several sizes and doses of zinc nanoparticles on antioxidant defense system in rat compared to controls. **METHODS:** Zinc nanoparticles (10, 20 and 30 nm) at 3 doses (3, 10 and 100 mg/kg bw) were administrated orally for 28 days among 9 experimental groups (n=5). One experimental group was treated orally with ZnCl2 (100 mg/kg bw) for 28 days and control group received normal saline (n=5). After 28 days, the rat was decapitated and serum was separated from the blood samples. The ferric reducing ability of Plasma (FRAP), thiobarbituric acid reactive substances (TBARS) and activity of glutathione peroxidase (GPx), and superoxide dismutase (SOD) enzymes in serum samples were measured as biomarkers of oxidative stress and compared with control group. **RESULTS:** This survey showed that zinc nanoparticles cause induction of GPx and SOD activity ($p<0.05$) and also increased the level of TBARS ($p<0.05$). This assay also showed zinc nanoparticles cause significant decrease in total antioxidant activity of plasma (FRAP) ($p<0.001$). **CONCLUSIONS:** Nano zinc induced oxidative stress in a dose dependent manner in large sizes, while their effects depend on the level of ionization in small sizes.

Introduction

Zinc (Zn) is one of the most important essential elements in the body of animals and plants. Zinc plays a significant role in the structure of more than 300 different proteins, metalloenzymes and transcription factors. Zinc is involved in many life supporting biochemical and metabolic processes such as the metabolism of protein, lipid, and carbohydrates, cellular respiration, detoxification of free radicals, and protection against lipid peroxidation (Hendy et al., 2001; Yousef et al., 2002; Carlson et al., 2004; Prasad, 2009; Frassinetti, 2006).

Zinc deficiency and the increase in the

production of reactive oxygen species lead to the generation of free radicals and lipid peroxidation in the tissues. A wide range of physiologic defects including disorders of the skin, growth retardation, and impaired neurologic, reproductive and immune systems are associated with zinc deficiency. Zinc deficiency alters the activities of some enzymes such as copper/zinc superoxide dismutase (Cu-Zn SOD). As an antioxidant, zinc inhibits the induction of oxidative stress through protecting sulfhydryl groups of proteins against free radicals, reducing the formation of free radicals by protective mechanisms (Jomova and Valko, 2011; Valko and Morris, 2005; Prasad, 2008).

Zinc nanoparticles (zinc NPs) are particles between 1 and 100 nanometers in size. Increasing the surface area of the particles changes the pressure and surface properties, viscosity and magnetic properties of the particles, leading to a change in the distance between the particles or their atoms, an increase in the ionization potentiality as well as a change in chemical reactions of the matter. Numerous applications of zinc NPs paved the way for oral, dermal and respiratory contacts with them. Oral contact with zinc nanoparticles happens through zinc supplements in the livestock food and food packaging. Dermal contact occurs through sunscreen, cosmetics, paint, paper and plastics. Respiratory contact happens in working environments (paint and nanoparticle producing factories). Despite the commercial production and widespread applications of zinc nanoparticles the safety of zinc NPs for humans, animals and other biological systems is still a controversial problem (Vandebriel and De Jong, 2012).

Some previous studies suggested that zinc nanoparticles are safe and revealed protective effects of zinc NPs against oxidative injuries (Afifi et al. 2015; Dawei et al., 2009; Malekshahinia et al., 2012). However, some other studies showed that exposure to zinc NPs resulted in oxidative stress and other adverse effects on animal and human health as well as cell cultures (Yousef, 2015; Xiong et al., 2011; Xia et al., 2008; Li et al., 2012). Some other studies showed that dissolved zinc ions induced metallothionein synthesis, and enhanced cellular resistance to oxidative stress. However, at higher doses zinc ions induced oxidative stress injuries. This suggested that different oxidative response mainly depend on the effect of size, dose, duration and route of exposure of zinc NPs (Zhang et al., 2012; Hejazy et al., 2012)

The present study aims to investigate the effects of subacute oral exposure to different sizes and doses of zinc NPs in comparison to bulk zinc on the oxidative stress parameters in rats.

Materials and Methods

Characterization of Zinc nanoparticles: Zinc NPs powder with 99.9 % purity, grey color, approximate concentration 0.2-0.4 g/m3 and specific surface area of 0.2-0.4 g/m3 was bought from Nanoshel Company. This powder is provided from zinc metal with high purity via the process of vaporization. The process of vaporization produces zinc with high purity, very small, very reactive and very reactant particles. The size and shape of the particles were determined by transmission electron microscopy (TEM). The particles were in three sizes of 10, 20 and 30 nm and they were mostly spherical.

Preparation of particle suspension: Prior to use, the particles were suspended in 1 % sodium carboxy methyl cellulose. The

particles were dispersed by ultrasonic vibration for 15 min, and some glass beads were added to avoid aggregation of the particles in the suspension (Wang et al., 2006).

Animals and treatment: 60 adult (10-week-old) male Wistar rats, weighing 253±25 g, were used in the study. During the whole experiment, animals were housed in controlled conventional conditions (temperature, 22±2 °C; relative humidity, 50-70 %; 12- h light-dark cycle). They were given free access to water and a conventional rodent pellet (2,390 kcal kg−1 metabolic energy and 10,320 kcal kg−1 digestible energy; crude protein, 19.5 %; crude fiber, 10 %; phosphor, 0.69 %; and calcium, 0.76 %).The design of experiments was approved by the local ethics committee. After a period of 2 weeks of acclimation, the rats were randomly divided into 9 experimental and control groups containing five animals each. The administrable zinc nanoparticles in each size (10, 20 and 30 nm) with 3, 10 and 100 mg/kg doses were mixed in 1% carboxy methyl cellulose solution by ultrasonic machine for 15 minutes. To prevent the aggregation of nanoparticles, glass globes were added to the suspension and it was subjected to vortex before every application (Wang et al., 2006). The administration of nanoparticles was done orally by gavage for 28 days.

. The control group received clean water without zinc plus carboxy methyl cellulose.

. Group 1-3: 3, 10 and 100 mg/kg doses of 10 nm Zinc NPs.

. Group 4-6: 3, 10 and 100 mg/kg doses of 20 nm Zinc NPs.

. Group 7-9: 3, 10 and 100 mg/kg doses of 30 nm Zinc NPs.

. Group 10: 100 mg/kg dose of bulk Zinc chloride.

At the end of the administration period the rats were anesthetized by chloroform and decapitated and blood sample was collected in the citrate-containing tubes. Blood serum was separated after centrifugation and kept at -80 oC until testing time.

Measurement of Oxidative Stress Biomarkers (Measurement of Plasma Total Antioxidant Capacity): Ferric Reducing Ability of Plasma (FRAP) method was used to assess plasma total antioxidant capacity. This method evaluates the ability of plasma in reducing ferric ions to ferrous. The basis of this assay is the formation of colorful complex of ferrous tripyridyltriazine [Fe (II)-TPTZ]. The amount of FRAP (micromol/liter) is achieved by comparing the absorption changes in 593 nm in the sample with solutions containing distinct concentrations of ferrous ion (Benzie and Strain, 1996).

Measurement of TBARS (ThiobarbituricAcid Reactive Substances): Plasma levels of MDA were estimated by the thiobarbituric acid reaction according to the method of Ledwoz et al. (1986). Briefly, 1 ml of plasma was mixed with 2 ml of freshly prepared thiobarbituric acid-trichloric acid-hydrochloric acid (TCA-TBA-HCl) reagent (30 g trichloroacetic acid, 0.75 g thiobarbituric acid and 4.2 ml concentrated HCl were mixed and diluted to 200 ml with distilled water) and 1.5 µl butylhydroxytoluene (0.05%). This mixture was boiled for 30 min. in a boiling water bath, and cooled to room temperature. n-Butanol extractable layer was centrifuged at 3000 ×g for 10 min., supernatant layer was removed and its absorbance was read at 535 nm. Concentrations of TBARS (nmol/mL) were determined from the standard curve using malondialdehyde bis (S4258497 537,

Table 1. FRAP, TBARS, SOD and Glutathione peroxidase levels in zinc and nano zinc treated groups. [a] Significant decrease ($p<0.05$) compared to control group. [b] Significant increase ($p<0.05$) compared to control group.

Treatment groups	FRAP (micromol/liter) (Mean±SD)	TBARS (nanomol/mL) (Mean±SD)	Superoxide dismutase(U/L) (Mean±SD)	Glutathione peroxidase (U/L) (Mean±SD)
Control group	1.761±0.0377	2.714±0.286	0.017±0.001	422.3±69.15
Zinc	1.855±0.1081	2.425±1.156	0.016±0.002	440.1±56.7
Nano Zn 10 nm (3mg/kg)	1.263±0.0915[a]	4.033±1.06[b]	0.019±0.005	175.4±48.8[a]
Nano Zn 10 nm (10mg/kg)	1.281±0.01513[a]	4.933±1.343[b]	0.017±0.001	246±35.44[a]
Nano Zn 10 nm (100mg/kg)	1.311±0.0118[a]	5.467±1.286[b]	0.022±0.003[b]	281.8±36.42[a]
Nano Zn 20 nm (3mg/kg)	1.08±0.1324[a]	3.393±1.097	0.012±0.001[a]	672±12.84[b]
Nano Zn 20 nm (10mg/kg)	1.307±0.445[a]	5.928±0.444[b]	0.015±0.002[a]	722.5±33.69[b]
Nano Zn 20 nm (100mg/kg)	1.395±0.4160	7.095±0.837[b]	0.026±0.001[b]	906.1±78.78[b]
Nano Zn 30 nm (3mg/kg)	1.579±0.0252[a]	4.333±0.650[b]	0.017±0.004	513±145
Nano Zn 30 nm (10mg/kg)	1.66±0.0816	4.367±0.611[b]	0.022±0.002[b]	618.5±183
Nano Zn 30 nm (100mg/kg)	1.644±0.346	7.933±0.404[b]	0.020±0.004[b]	870.2±93.21[b]

Merck Company, Tehran, Iran).

Measurement of Superoxide Dismutase (SOD): Superoxide Dismutase is involved in the detoxification of O2 toxic radical. In this method, Xanthine and Xanthine oxidase are used to produce superoxide radicals. They react with 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-tetrazolium chloride (INT) and red color of formazan is produced which is measured at 505nm wavelength. If SOD enzyme exists in the superoxide radicals sample, it is turned into hydrogen peroxide and O2, inhibiting the production of red color of formazan. The activity of SOD enzyme is determined by the degree of its inhibition of this reaction. One unit of SOD restrains the INT reduction speed up to 50% or restrains nicotinamide adenine dinucleotide phosphate (NADPH) oxidation up to 50% under the measurement concentrations. SOD was done by using the commercial kit of Ransod (Randox) on the basis of colorimetric method with some modifications.

Measurement of Glutathione Peroxidase: This method is based on the method introduced by Valentine and Paglia (1967). Glutathione peroxidase enzyme catalyses glutathione oxidation reaction (GSH) by Cumenehydroperoxide. In the presence of glutathione reductase and NADPH, oxidized glutathione (GSSG) turns into reduced glutathione again and this reduction is simultaneous with oxidation of NADPH into NADP+. In this reaction, light absorption reduction is measured at 340 nm wavelength. Glutathione peroxidase measurement was done by using the commercial kit of Ransod (Randox) on the basis of enzymatic method with some modifications.

Statistical analysis: Statistical analysis was done using Graph Pad InStat, version 3.06 (Graph Pad Software, Inc). The measures were expressed according to Means ± SD. T test analysis was performed to show significance between control and others groups. $p<0.05$ was considered statistically significant.

Results

In nano zinc (10 nm) treated groups, the amount of FRAP significantly decreased compared to the control group ($p<0.05$), it seems that the amount of FRAP has increased by increasing the doses and sizes of zinc nanoparticles (Table 1). As shown in

Table 1, it seems that the amount of malondialdehyde has increased by increasing the administered dose of zinc nanoparticles. The administration of 10 nm zinc nanoparticles significantly decreased ($p<0.0001$) the amount of GPx compared to controls. The administration of 20 and 30 nm zinc nanoparticles in all doses increased the amount of GPx ($p<0.0001$) in a dose-dependent manner (Table 1).

Discussion

Previous studies showed the antioxidative effects of bulk zinc (Powell and Saul 2000; Bray et al., 1990; Prasad and Anada, 2004; Rostan and Elizabeth, 2002; Zago et al., 2001; Sun et al., 2006). As shown in our study, in contrast to the bulk zinc, nano zinc remarkably decreased plasma total antioxidant capacity, especially in lower sizes. It can be due to the fast release of the high amount of Zn2+ion from the administered zinc nanoparticles (Reed, 2012). Previous studies have shown that fast solubility of Zn2+ion of nano zinc in the cell and other biological systems leads to the fast access of the cells and the biological systems to zinc ions (Deng, 2009; Ma et al., 2012). Zn 2+ seems to be responsible for inducing oxidative stress.

In groups which received the smallest sizes of nanoparticles (10 nm), the amount of MDA increased whereas the amount of GPx and FRAP decreased. The lower levels of GPx along with the decrease of FRAP could cause more oxidative stress effects and raise the amount of MDA. In higher doses and sizes SOD and GPx levels increased. These observations give rise to the hypothesis that nanoparticles of smaller sizes can induce more potent oxidative stress damages as other studies reported that the high toxicity of nanoparticles in cells increases with the reduction of size (Hanley et al., 2009; Wang et al., 2008; Cho et al., 2011). However, Guo et al. (2008) showed that the toxic effects of zinc nanoparticles in leukemia cells are related to their surface structure and dose-dependent effects are insignificant.

Compared with Wang et al.'s (2006) and (2008) studies, it seems that smaller sizes of nanoparticles with lower doses and larger size nanoparticles with higher doses produce more toxic effects. It seems release of Zn+2 ions in biological solutions is more convenient at lower sizes and doses of nanoparticles, while in higher doses and sizes of nano zinc, aggregation of nanoparticles decrease the release of Zn+2 ions and toxic effects.

Some other studies on the laboratory animals and cell culture mediums showed time dependent toxicity of zinc NPs. The duration of exposure to nano zinc plays an influential role in oxidative stress induction and also defensive responses (Bakhshiani and Fazilati, 2014; Trevisan, 2014; Valdiglesias et al., 2013). It seems that antioxidant defense system is induced gradually during the time of exposure to adapt animals against the nanoparticles adverse effects of oxidative injury (Bakhshiani and Fazilati, 2014; Trevisan, 2014). Some studies reported induction and activation of SOD and metallothioneins after nano zinc administration, while significant expressions of metallothioneins by larger sizes and higher doses of nano sized zinc were observed in our complementary studies (Xiao-bo et al., 2009; Hejazy et al., 2014). However, Zhang et al. (2012) reported that at low concentration of nano zinc, dissolved zinc ions induced metallothionein synthesis, enhanced cellular resistance to

oxidative stress. At higher doses, excessive zinc destroyed mitochondrial function and cell membrane and caused cell necrosis of mouse alveolar macrophages (MH-S).

Similar to our study, some in vitro and in vivo studies showed that oxidative stress has a principle role in nano zinc induced cytotoxicity (Ahamed et al., 2011; Lenz et al., 2009; Huang et al., 2010; Kim et al., 2010; Osmond and McCall, 2010; Kao et al., 2012; Cho et al., 2011). Yousef and Mohamed (2015) reported increase in malondialdehyde (MDA), decrease in glutathione peroxidase (GPx) of rat liver tissue in response to oral administration of 500mg/kg nano zinc particles for 10 days. Zhao et al. (2013) showed acute ZnO nanoparticles exposure induces developmental toxicity, oxidative stress and DNA damage in embryo-larval zebra fish. Surekha et al. (2012) showed a significant decrease in collagen content and oxidative stress with an inverse dose relationship in nano zinc oxide-treated rats. Sharma et al. (2012) reported induction of oxidative stress, DNA damage and apoptosis in mouse liver after sub-acute oral exposure to zinc oxide nanoparticles. Wong et al. (2010) reported significant up-regulation of SOD, MT and HSP70 and oxidative stress in nano zinc oxide treated marine organisms.

Lina et al. (2009) reported that supplemental zinc oxide in broilers chicken significantly increased the activity of glutathione peroxidase and serum antioxidant and decreased MDA content in serum and liver of chickens. They also reported decrease in serum Nitric oxide and Hydroxyl radicals and increase in the activity of resisting superoxide anion free radical in liver. Decrease of SOD, catalase and GSH levels and increase in MDA content in the kidney, spleen and heart of mice treated with the zinc oxide nanoparticles was reported by Fang et al. (2010).

However, some studies concluded protective effects of nano zinc against oxidative stress. Malekshahinia et al. (2012) reported that endurance exercise induced oxidative stress in the male reproductive system and can be protected by nano zinc oxide supplementation. Afifi et al. (2015) showed significant decrease in the MDA levels and significant increase in the activity and mRNA expression of SOD, CAT, GPx, GRD, and GST, in testicular tissue of diabetic rats treated with Zinc oxide NPs. Dawei et al. (2010) revealed protective effects of nano zinc on the primary culture mice intestinal epithelial cells against oxidative injury.

Nano zinc particles are expected to be more toxic than their bulk ones because of their greater surface reactivity and their capacity to penetrate into cells and organisms (Ispas et al., 2009; Mironava et al., 2010). Dissolved Zinc ions increase in the cells, leading to increase of intracellular ROS generation, membrane damage, Ca2+flux and mitochondrial activity impairment, apoptosis, inhibition of mitochondrial respiratory chain. ROS generation leads to oxidative stress and in consequence, lipid peroxidation and oxidative DNA damage (Xiong et al., 2011; Vandebriel & De Jong, 2012).

As reported in different studies, our study showed change of oxidative stress parameters in treated animals. However, different results in various studies may relate to different physicochemical properties of the nanoparticles such as size, surface shape, agglomeration property, liberation and solubility. Moreover, exposure duration, animal species, administration route may have some effects on oxidative stress parameters.

Dose response relationship of Zinc nanoparticles must be investigated in more detail. In some cases inverse dose dependency effects were reported. Therefore, more experiments are required to understand the dose -response and size-response relationship of nano Zinc. New concept of dose metric that was introduced in nanotoxicology should be further investigated.

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مجله طب دامی ایران، ۱۳۹۶، دوره ۱۱، شماره ۲، ۱۶۳-۱۵۵

بررسی اثرات مواجهه تحت حاد با نانو ذرات روی بر شاخص‌های استرس اکسیداتیو در رت

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(دریافت مقاله: ۳۰ آبان ماه ۱۳۹۵، پذیرش نهایی: ۱۹ بهمن ماه ۱۳۹۵)

چکیده

زمینه مطالعه: روی یکی از ضروری ترین عناصردر بدن حیوانات و گیاهان به شمار می‌رود. روی نقش مهمی در ساختار بیش از ۳۰۰ پروتئین مختلف و بسیاری از فرایندهای بیوشیمیایی و متابولیک مانند متابولیسم، تنفس سلولی و محافظت در برابر رادیکال‌های آزاد ایفاء می‌کند. نانوذرات روی نوع جدیدی از روی است که در مواد آرایشی و بهداشتی و غذای حیوانات و بسته بندی مواد غذایی کاربرد دارد. **هدف:** ارزیابی اثرات دوزها و سایزهای مختلف نانو روی بر سیستم دفاع آنتی اکسیدانی در رت است. **روش کار:** نانو ذرات روی در سه سایز (۳۰، ۲۰، ۱۰ nm) و در سه دوز ۳، ۱۰، ۱۰۰ mg/kg bw به مدت ۲۸ روز به ۹ گروه آزمایش تجویز شدند. به یک گروه کلرید روی ۱۰۰ mg/kg bw و به گروه کنترل نرمال سالین تجویزشد. پس از ۲۸ روز رتها سر بریده شدند و سرم خون آنها جدا شد و میزان TBARS، FRAP و میزان فعالیت SOD وGPx به عنوان بیومارکرهای استرس اندازه‌گیری شد. **نتایج:** نتایج نشان داد که نانوذرات روی باعث القای فعالیت SOD وGPx، افزایش میزان TBARS و کاهش میزان خاصیت آنتی اکسیدانی تام پلاسما FRAP می شود ($p<۰/۰۵$). **نتیجه‌گیری‌نهایی:** نانوروی در سایزهای بزرگ به صورت وابسته به دوز و در سایزهای کوچک بسته به میزان حلالیت پذیری باعث القای اکسیداتیو استرس می‌شود.

واژه های کلیدی: FRAP، GPx، نانو روی، اکسیداتیو استرس، رت

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