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Effects of aluminum, copper, and titanium nanoparticles on some blood parameters in Wistar rats

Esin Gülnaz CANLI, Mustafa CANLI*
Department of Biology, Faculty of Sciences and Letters, Çukurova University, Adana, Turkey

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Abstract: In this study, Al_2O_3, CuO, and TiO_2 nanoparticles (NPs) were individually administered to 60 mature female rats via oral gavage (0, 0.5, 5, and 50 mg/kg b.w. per day) for 14 days and then responses of ATPases in the erythrocytes, total oxidant status (TOS), total antioxidant status (TAS), and liver enzyme (alkaline phosphatase, ALP; alanine transaminase, ALT; aspartate transaminase, AST) levels in the serum were determined. There were sharp decreases in Na,K-ATPase activity in the erythrocytes following NP exposures. All doses of CuO caused significant ($P < 0.05$) inhibitions in Na,K-ATPase activity (up to 94%), while only higher doses of Al_2O_3 and TiO_2 inhibited (76%–80%) the activity of Na,K-ATPase. Oppositely, the activities of Ca-ATPase (up to 274%) and Mg-ATPase (up to 290%) increased significantly following TiO_2 exposures. TOS levels significantly increased following Al_2O_3 (167%) and CuO (240%) exposures, though TAS levels did not change significantly in any of the exposure groups. The levels of ALP, AST, and ALT increased following NP administrations. Ti-NP increased the levels of all liver enzymes in the serum (up to 84%), while Al-NP (58%) and Cu-NP (43%) increased only ALP levels. The present study demonstrated evidence of the toxic effects of NPs, as they altered the measured parameters in the blood, and suggests further research to better understand the environmental fate of NPs.

Key words: Nanoparticles, rat, erythrocyte, serum, toxicity

1. Introduction

Following recent technological developments, nanosized (<100 nm) metal complexes have entered into different areas of human lives and the discussion of their potential effects on humans and the environment has begun (Jeng and Swanson, 2006; Janrao et al., 2014). The areas in which nanoparticles (NPs) are used for anthropogenic purposes include medicine, the textile and electronic industries, filters, toothpaste, suntan cream, toys, moisturizers, packing products, household appliances, and the food industry. Although they are widely used, there are no satisfactory data on the environmental fate of these particles, despite vigorous attempts to find out their possible effects in different groups of animals. Those studies indicated that NPs may possess health hazard effects, domestically, medically, and environmentally, due to their high surface-to-volume ratio, small size, crystallinity, electronic properties, surface structure, reactivity, functional groups, inorganic or organic coatings, shape, and aggregation behavior, which can make the particles very reactive or catalytic (Keller et al., 2010; Schrand et al., 2010; Janrao et al., 2014). NPs are able to pass through cell membranes and their interactions with biological systems are relatively unknown (Ahamed et al., 2010; Jeng and Swanson, 2014). Therefore, NPs can potentially cause adverse effects on organs, tissues, cellular and subcellular functions, and enzymes due to their unusual physicochemical properties (Keller et al., 2010; Schrand et al., 2010). Studies have shown that NPs have toxic effects on mammals depending on metal type, size, dose, and administration route of these particles (Elle et al., 2013; Wang et al., 2013; Jeng and Swanson, 2014). Although the literature data present so far are not consistent and do not show clear evidence of the toxicity of NPs, it seems they are not innocent products of mammalian metabolism. Biomarkers belonging to different metabolic systems in mammals were altered by NPs administrated via the oral route, inhalation, or subcutaneous injection (Sha et al., 2011; Syama et al., 2013; Shrivastava et al., 2014; Heydrnejad et al., 2015; Hu et al., 2015; Lei et al., 2015).

Osmoregulatory enzymes, ATPases, are membrane-bound enzymes responsible for the transport of ions through cell membranes and thus play significant roles in the regulation of cell volume, osmotic pressure, and membrane permeability (Monserrat et al., 2007; Atli and Canli, 2011). Na,K-ATPase transports 3 Na ions out of the

* Correspondence: mcanli@cu.edu.tr

cell and 2 K ions into the cell to maintain the ionic balance, while Mg-ATPase plays an important role in oxidative phosphorylation and ionic transport and is responsible for transepithelial regulation of Mg ions (Canli et al., 1996; Parvez et al., 2006). Ca-ATPase removes Ca ions from the cytoplasm for protecting the low Ca levels in the cell (Saxena et al., 2000). Osmoregulation system enzymes are sensitive to xenobiotics (e.g., metals), as they are able to bind onto the active sites of enzymes. For this reason, inhibition or stimulation of ATPases was shown in the literature after exposing animals to metals or NPs (Canli and Stagg, 1996; Griffitt et al., 2007; Atli and Canli, 2011, 2013; Guo et al., 2013; Singh et al., 2013).

Oxidative stress is potentially experienced by all aerobic life when antioxidant defenses are overcome by prooxidant forces and is the basis of many physiological aberrations (Winston, 1991). Antioxidant system parameters include both enzymatic and nonenzymatic elements to eliminate oxidants produced by the organism itself or environmental toxicants such as reactive oxygen species (ROS), metals, pesticides, and NPs. Antioxidant enzymes, such as superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR), and catalase (CAT), are crucial in the effort to counteract oxidative stress caused by toxicants once the supply of other antioxidant compounds is depleted (Atli and Canli, 2008; Yilmaz et al., 2015). In certain conditions, an increase in oxidant levels and a decrease in antioxidant system elements may occur, causing oxidative stress due to changes in the oxidative/antioxidative balance. One of the rapid determination methods of total oxidants and antioxidants in body fluids is to measure total oxidant status (TOS) and total antioxidant status (TAS), which have been used in numerous papers (Akalin et al., 2007; Koksal and Kurban, 2010; Wei et al., 2010; Ogut et al., 2015).

Liver enzymes such as alkaline phosphatase (ALP), alanine transaminase (ALT), and aspartate transaminase (AST) found in the serum are considered to be important serum markers to investigate the health of animal species of concern (Tang et al., 2011). ALP is a polyfunctional enzyme that acts as a transphosphorylase at alkaline pH levels and plays a pivotal role in the mineralization of the skeleton of animals. In addition, two major aminotransferases, AST and ALT, are the most significant enzymes involved in protein and amino acid metabolism. ALP, ALT, and AST are serum enzymes that are commonly used as important serum biomarkers to investigate the health of animals (Zhang et al., 2011).

Al_2O_3, CuO, and TiO_2 NPs are the most abundantly produced nanomaterials and have been used in diverse fields, including the medical, military, chemical, electronics, biomedicine, cosmetics, and food sectors (Klaine et al., 2008; Janrao et al., 2014). Once they are used for different purposes, these NPs will inevitably enter the environment and may cause adverse effects for aquatic or terrestrial organisms. The effects of TiO_2, ZnO, and AgO NPs on mammalian metabolism have been studied much more compared to CuO or Al_2O_3 (Ahamed et al., 2010; Jeng and Swanson, 2014). To the best to our knowledge, there are not sufficient data on the effects of these NPs in rat blood, and especially on ATPase activities in erythrocytes.

2. Materials and methods

2.1. Experimental protocol

This study was carried out under the ethical approval (6.4. 27.07.2015) of the Medical Sciences Experimental Research and Application Center (DETAUM) of Çukurova University. Wistar albino Sprague Dawley rats (*Rattus norvegicus* var. albinos) are reproduced continuously at DETAUM. All the experiments were carried out at this center where the animals were reproduced. Only female rats were used in the experiments. The rats were allocated into 7 cages applying a photoperiod of 12 h of light at 22 ± 1.5 °C and the moisture of the laboratory was kept between 48% and 56%. During the experiments, rats were fed with standard rat food. Weight of rats ranged between 190 and 220 g and there was no significant difference ($P > 0.05$) among different exposure groups. Each experimental group consisted of 6 rats and a total of 60 rats were used in the experiments, including a control group. NPs were purchased from Sigma-Aldrich (Germany) or Nanografi (Turkey). NP suspensions were mixed vigorously, sonicated for 20 min on ice prior to the experiment, and immediately applied to the related assay to minimize agglomeration.

Rats received Al_2O_3 (40 nm), CuO (40 nm), and TiO_2 (21 nm) NPs in 200 µL water via oral gavage (0, 0.5, 5, or 50 mg/kg b.w. per day), while controls received only the same amount of water. All the experiments were ended after 14 days. After this period, rats were taken out of the cages, killed with high doses of anesthesia (Ketasol 10%), and dissected carefully using sterile equipment. Blood was removed from the heart using syringes and put into glass tubes. Blood samples were centrifuged at 3000 × *g* (Hettich Universal 30 RF, Germany) for 5 min (4 °C) to separate the serum and the cells. Serums were removed and the cells were washed three times with 0.09% NaCl.

2.2. Analyses of blood parameters

ATPase activities in the erythrocytes were measured using the method of Atkinson et al. (1973). Specific Na,K-ATPase activity was calculated from the inorganic phosphate liberated from ATP using the differences between the presence (Mg-ATPase activity) and absence (total-ATPase activity) of ouabain. Ca-ATPase activity was measured as the absorbance differences between the presence and absence of $CaCl_2$ using the same method. Details are

given in our previous paper (Atli and Canli, 2011). Serum parameters were analyzed immediately in the central laboratory of Balcalı Hospital (Adana, Turkey), which was accredited by the Joint Commission International for a third time until January 2017. The IgG, IgE, and IgM levels in the serum were measured by nephelometric methods (Thomas, 1998; Winter et al., 2000) using a Siemens BN II nephelometer. Appropriate kits were supplied by Siemens Company (Germany). The liver enzymes (ALP, ALT, AST) found in the serum were analyzed using a DXC 800 Beckman Coulter autoanalyzer (Newman et al., 1992; Young and Bermes, 1999). Reactants for all the measurements were supplied by Beckman Coulter Company (USA). Analyses of TOS and TAS were carried out spectrophotometrically (Shimadzu UV-1800) using the kits of Rel Assay Diagnostics (Turkey). The methods described by Erel (2004, 2005) were used to measure TAS and TOS levels in the serum.

2.3. Statistical analyses

SPSS 15 (SPSS Inc., Chicago, IL, USA) was used for the analysis of data. Data for the serum and erythrocyte parameters were presented as mean and standard error of the mean. Before the statistical analysis, homogeneity of variance was checked among different exposure periods to evaluate the distribution of data. One-way ANOVA testing was applied to data if they were normally distributed; otherwise, the Kruskal–Wallis test was applied.

3. Results

This study showed that 14-day oral administration of Al_2O_3, CuO, and TiO_2 NPs (up to 50 mg/kg per day) to female rats was not considerably lethal. There were 3 mortalities (5%) out of 60 rats and those mortalities occurred at the lowest (0.5 mg/kg per day) NP doses (1 for each NP).

Figures 1–3 show the activities of Na,K-ATPase, Mg-ATPase, and Ca-ATPase in the erythrocytes following NP administrations. Mean activities and associated standard errors of Na,K-ATPase, Mg-ATPase, and Ca-ATPase in controls were 1.55 ± 0.36, 3.10 ± 0.62 and 1.26 ± 0.35 μmol Pi/mg protein per hour, respectively. All NPs at all doses significantly decreased the Na,K-ATPase activity. Oppositely, the activities of Mg-ATPase and Ca-ATPase significantly increased, but only at the higher doses of TiO_2. Mean TOS and TAS levels and associated standard errors in the serum of control rats were 9.29 ± 0.89 mmol/L and 1.38 ± 0.52 μmol/L, respectively. TOS levels did not change significantly in the TiO_2 NP-exposed group, but it increased significantly in the Al_2O_3- and CuO-exposed groups (Figure 4). TAS levels, however, did not change significantly in any of the NP-exposed rats (Figure 5). The mean ALP activity and associated standard error in the serum of control rats was 185 ± 17 U/L. All NPs significantly increased ALP activity (Figure 6). ALP activities were 293 ± 24, 265 ± 23, and 251 ± 12 U/L in rats after Al, Cu, and Ti NPs exposure, respectively. The mean ALT activity and associated standard error in the serum of control rats was 226.3 ± 73.8 U/L (Figure 7). Significant alteration only occurred in Ti-NP-exposed rats, as levels increased to 357 ± 72 U/L. Similarly, AST activity altered significantly in Ti-NP-exposed rats, as the activity increased to 796 ± 103 U/L from a control value of 516 ± 143 U/L (Figure 8). Percent alterations and associated statistical results of the studied parameters are given in the Table.

4. Discussion

The current experiments showed that Al_2O_3, CuO, and TiO_2 NPs administered orally to Wistar rats at doses of 0.5, 5, and 50 mg/kg per day could be accepted as sublethal for 14 days, as there was only 5% rat mortality. The death of

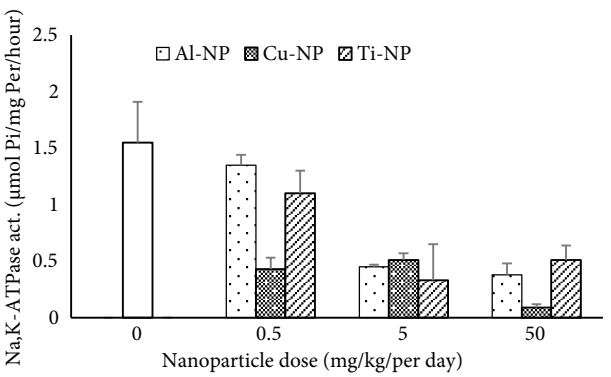

Figure 1. The activity of Na,K-ATPase in the erythrocytes of female rats orally exposed to Al_2O_3, TiO_2, and CuO nanoparticles for 14 days. Each point shows the mean of 6 rats and the standard errors. Statistical results and % alterations are given in the Table.

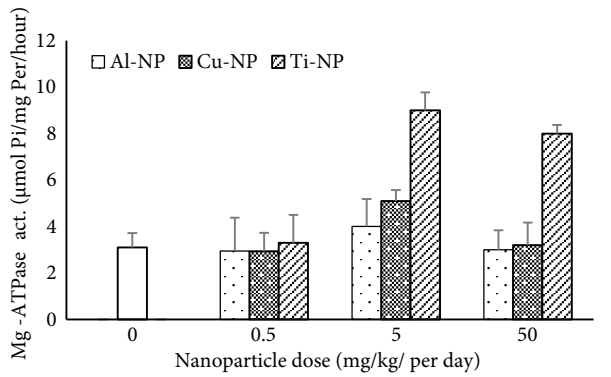

Figure 2. The activity of Mg-ATPase in the erythrocytes of female rats orally exposed to Al_2O_3, TiO_2, and CuO nanoparticles for 14 days. Details are given in Figure 1.

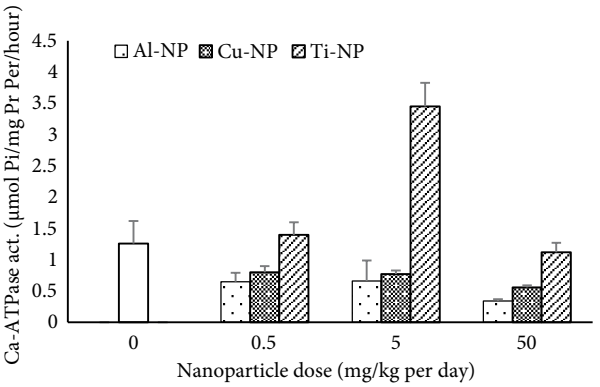

Figure 3. The activity of Ca-ATPase in the erythrocytes of female rats orally exposed to Al_2O_3, TiO_2, and CuO nanoparticles for 14 days. Details are given in Figure 1.

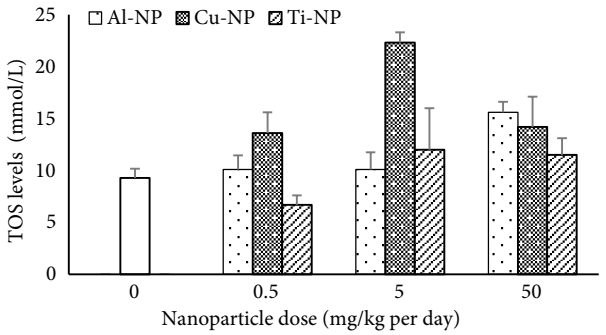

Figure 4. Total oxidant levels in the serum of female rats orally exposed to Al_2O_3, TiO_2, and CuO nanoparticles for 14 days. Details are given in Figure 1.

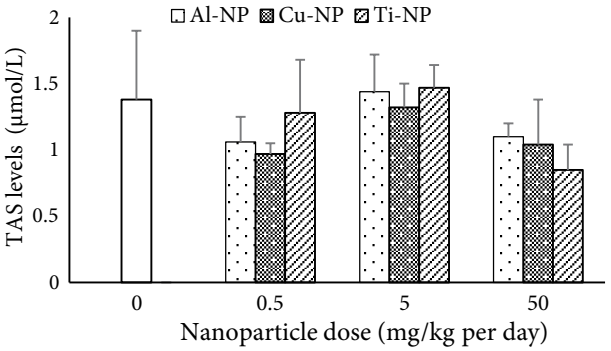

Figure 5. Total antioxidant levels in the serum of female rats orally exposed to Al_2O_3, TiO_2, and CuO nanoparticles for 14 days. Details are given in Figure 1.

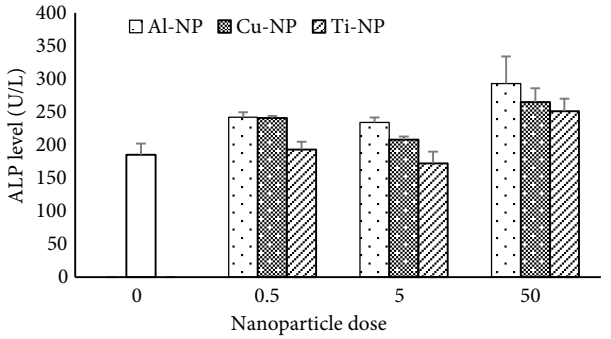

Figure 6. ALP levels in the serum of female rats orally exposed to Al_2O_3, TiO_2, and CuO nanoparticles for 14 days. Details are given in Figure 1.

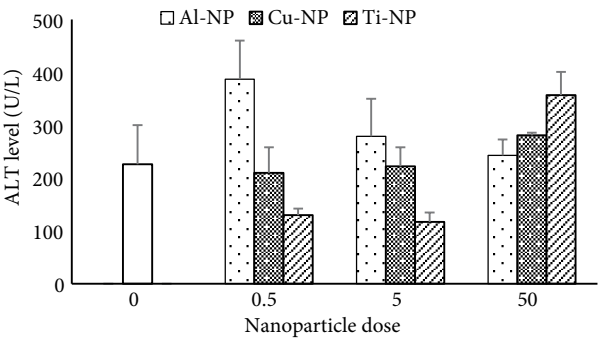

Figure 7. ALT levels in the serum of female rats orally exposed to Al_2O_3, TiO_2, and CuO nanoparticles for 14 days. Details are given in Figure 1.given in Figure 1.

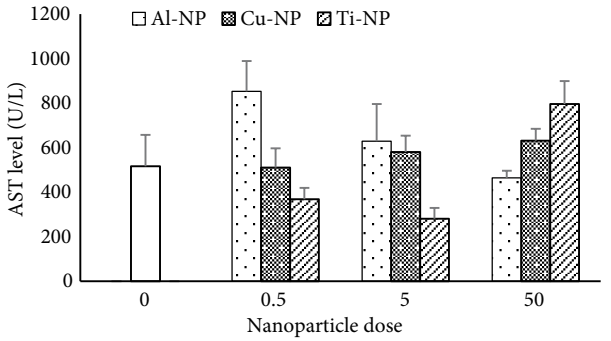

Figure 8. AST levels in the serum of female rats orally exposed to Al_2O_3, TiO_2, and CuO nanoparticles for 14 days. Details are given in Figure 1.

these rats occurred at the lowest doses of NPs, indicating that rat mortality was due to natural causes rather than NP administrations. Additionally, there were no apparent health problems (e.g., changes in movement, appetite, or eye color) in the rats. In general, lethal doses of different metal NPs for rats are relatively high compared to dissolved metals (Xia et al., 2009; Schrand et al., 2010; Janrao et al., 2014). One of the reasons for this may be the strong affinity of dissolved metals to bind to essential molecules and enzymes active sites (Heath, 1995; Jorgensen, 2010).

Table. Percent alterations in the studied parameters in the blood of female rats after 14-day oral administrations of Al_2O_3, CuO, and TiO_2 nanoparticles. The differences in mean values between control group and NP-exposed groups were used in % calculations as given in the table. Upward arrows indicate significant increases and downward arrows indicate significant decreases. Only significant differences ($P < 0.05$) are given in this table.

Parameters	Al_2O_3 NP doses (mg/kg per day)			CuO NP doses (mg/kg per day)			TiO_2 NP doses (mg/kg per day)		
	0.5	5	50	0.5	5	50	0.5	5	50
Na,K-ATPase		↓ 71	↓ 76	↓ 63	↓ 67	↓94		↓80	↓68
Mg-ATPase								↑ 290	↑ 264
Ca-ATPase								↑ 274	
Total oxidants			↑ 167		↑ 240				
Total antioxidants									
ALP			↑ 58			↑ 43			↑ 35
ALT									↑ 84
AST									↑ 54

4.1. Osmoregulation system enzymes

Ion-transporting ATPases are very sensitive to environmental contaminants for both terrestrial and aquatic organisms (Zhang et al., 1990; Canli and Stagg, 1996; Carfagna et al., 1996; Atli and Canli, 2011, 2013). This assumption was supported by the present study, as ATPase activities in the erythrocytes showed the strongest alterations compared to the other parameters studied. Na,K-ATPase activity decreased sharply in all NPs and almost all NP doses. However, Mg-ATPase and Ca-ATPase activities oppositely increased after TiO_2 exposures, though there were no significant alterations after Al_2O_3 and CuO exposures. NPs are able to penetrate very small capillaries throughout the body, causing accumulation in tissues. During this movement, NPs pass through epithelia and biological membranes, which can eventually cause toxic effects on the physiology of cells (Schrand et al., 2010). The response of ATPases may differ greatly against metal exposures considering in vitro and in vivo exposure protocols (Vasic et al., 2009). Although dissolved metals generally inhibit ATPases in vitro, inhibition of these enzymes by metals in vivo can be compensated by homeostatic regulation and their activity may return to normal or can be stimulated (Atli and Canli, 2011, 2013). This recovery may possibly occur by increasing the number of the enzyme molecules and/or increasing the turnover rates of enzymes present in order to compensate the activity of lost enzymes. However, this assumption cannot be applied to metal-containing NPs, because there are not sufficient data on the effects of metal NPs on mammalian ATPases. A few studies have shown different conclusions on the effects of NPs. Petrovic et al. (2012) studied the influence of gold NPs on Na,K-ATPase and Mg-ATPase activity in rat brain synaptosomal plasma membranes. They found that gold NPs induced the stimulation of Na,K-ATPase activity by more than 100% with no change in Mg-ATPase activity. They concluded that stimulation of enzyme activity was a consequence of an increase of the active surface of membranes. Chichova et al. (2014) studied the possible influence of Ag NPs on the activity of rat liver mitochondrial ATPase and found that Ag NPs inhibited ATPase activity in the mitochondria. This suggests nanoparticle aggregation and thus a possible reduction in their reactivity. Guo et al. (2013) demonstrated that Zn NPs decreased the expression and activity of plasma membrane Ca-ATPase, causing disruption of the intracellular calcium homeostasis in rat retinal ganglion cells. They concluded that the disrupted calcium homeostasis would trigger mitochondrial dysfunction, generate excessive ROS, and finally initiate cell death. Wang et al. (2013) showed that Zn NPs inhibited Ca-ATPase expression in human lens epithelial cells under ultraviolet irradiation. Kumari et al. (2013) studied the acute oral effects of high doses of Fe NPs in rats. They found that Fe NPs caused significant inhibition of acetylcholinesterase in the red blood cells, as well as in the brains. Furthermore, there was more than 50% inhibition of Na,K-ATPase, Mg-ATPase, and Ca-ATPase, as observed in brains of exposed female rats. The authors suggested that this situation might be the result of disturbances in cellular physiology and the ionoregulatory process. The present study produced clear evidence of the inhibition of Na,K-ATPase activity following oral administration of Al_2O_3, CuO, and TiO_2 NPs. The erythrocyte is a single cell with a large surface

area and thus it faces contaminants flowing in the blood. NPs may stick or bind onto the membrane of erythrocyte and thus may inhibit the transport of Na and K ions by Na,K-ATPase. Ca-ATPase and Mg-ATPase are not located in the cell membrane and therefore they were possibly less affected by NPs bound on the cell membrane.

4.2. Total oxidant and antioxidant status

The antioxidant system contains several enzyme systems that catalyze reactions to neutralize free radicals and ROS. These enzymes include SOD, CAT, GR, and GPx. Animals also maintain complex systems of multiple types of antioxidants, such as glutathione, vitamin C, vitamin A, and vitamin E. Antioxidants inhibit the oxidation of other molecules in the metabolism that are necessary for chemical reactions involving the loss of electrons or an increase in oxidation state. Many studies showed that metals can induce oxidative stress by generating free radicals and ROS, and the liver and kidney have antioxidant defense systems to protect animals from oxidative stress caused by metals (Jorgensen, 2010). NPs were regarded as one of the main mechanisms involved in induced toxic manifestations in animals, as they can penetrate inside the cytoplasm and nucleus (Shrivastava et al., 2013). The present data demonstrated that total oxidant levels in the serum of rats increased considerably, though total antioxidant levels did not follow this trend, suggesting possible oxidative stress. It is known that insufficient levels of antioxidants or inhibition of antioxidant enzymes can cause oxidative stress and may damage or kill cells. Likewise, the expressions of specific lesions known to arise specifically from oxidative stress, e.g., lipid peroxidation, oxidized bases in DNA, and accumulation of lipofuscin pigments, are present in animals exposed to contaminants (Winston, 1991). Syama et al. (2013) showed that there was a concentration-dependent fall in cell viability, a decrease in antioxidant enzyme levels, and an increase in DNA adduct of the liver of mice following zinc NP exposures. Shrivastava et al. (2013) studied the effects of TiO_2, ZnO, and Al_2O_3 NPs on oxidative stress in the erythrocytes, liver, and brain of male mice following oral administration of NPs for 21 days. They found that there was significant production of ROS and consecutively antioxidant enzyme activities altered, supporting the present results. Their results and the present results also agree on the least toxic effects of TiO_2 NPs compared to other NPs. The TOS levels in TiO_2-exposed rats were also increased in the present study, but this was not statistically significant. In recent years, there has been considerable evidence in the literature on ROS increase and antioxidant system failure following TiO_2, CuO, ZnO, and Al_2O_3 exposures of rats (Sha et al., 2011; Yu et al., 2014; Hu et al., 2015; Lei et al., 2015), supporting the present data, which showed the TOS increase in the serum.

4.3. Liver enzyme levels

This study demonstrated that liver enzymes ALP, ALT and AST measured in the serum increased following oral administration of NPs, especially in rats exposed to Ti-NP. ALP activity was increased by all NPs, though ALT and AST levels increased only in rats exposed to Ti-NP. An increase in the levels of transaminases is related to damage of the liver, kidney, heart, and other tissues in the state of stress influenced by xenobiotics (Li et al., 2012). Therefore, data presented here suggest that the liver of rats, and especially those that received Ti-NP, may be damaged, as the liver is a major target of nanoparticle accumulation. The effects of aluminum oxide nanoparticles (50 mg/kg) and non-nano-aluminum oxide (50 mg/kg) in Sprague Dawley rats exposed subcutaneously to these two forms of aluminum showed that the levels of ALT and AST in the plasma were significantly higher in the nano-aluminum oxide group than the non-nano-aluminum oxide and control groups, suggesting damage of the liver (Li et al., 2012). Sharma et al. (2012) also indicated that the liver is the primary organ of metabolism and might act as a major target organ for nanoparticles after they gain entry into the body through any of the possible routes. Their study showed that ALP and ALT levels increased in the serum of mice that received Zn-NP (300 mg/kg) for 14 days, demonstrating pathological lesions in the liver. Male rats orally administered Ag-NP with doses between 5 and 100 mg/kg for 5 days had increases in ALP, ALT, and AST levels, especially at higher doses (Patlolla et al., 2015). Similarly, increases in ALP and AST levels were also found by Tang et al. (2011) in the serum of rats exposed to Ti-NP by intratracheal instillation at low (0.8 mg/kg), medium (4 mg/kg), and high (20 mg/kg) doses. They also concluded that the increase in levels of these liver enzymes may be due to possible liver damage. Zhang et al. (2011) showed the in vivo toxicity of different sizes of gold nanoparticles in mice. They found that mice exposed to gold NPs (10 and 60 nm) with a dose of 4 mg/kg over 28 days had a significant increase in ALT and AST levels, indicating damage to the liver. As shown above, the present data and the literature data are in agreement about the toxic effects of NPs to the liver, demonstrated by an increase in serum enzymes.

In conclusion, this study demonstrated that 14-day oral administration of TiO_2, CuO, and Al_2O_3 NPs (0, 0.5, 5, and 50 mg/kg b.w. per day) altered the osmoregulation, immune, and antioxidant system parameters as well as the activities of the liver enzymes (ALP, ALT, AST) in female rats. The most striking results were seen in ATPase activities of the erythrocytes. Na,K-ATPase activity in all NP exposures decreased sharply, whereas activities of Mg-ATPase and Ca-ATPase in the erythrocytes increased in TiO_2-exposed rats. There were increases in total oxidant levels in the serum of CuO- and $Al2O_3$-exposed rats, suggesting possible oxidative

stress. The levels of liver enzymes measured in the serum increased, especially in Ti-NP-exposed rats, suggesting liver damage. This study indicates the need to carry out further research to better identify the toxic effects of NPs in different groups of animals with different exposure routes, which would help us to better understand the environmental fate of these particles.

Acknowledgments

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