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Investigations on the Energy Reserves of Rat Liver Following Oral Exposures to Nanoparticles

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Abstract

The liver is the energy store of mammals and its energy budget can change due to xenobiotic stress. There is no adequate information on the effects of nanoparticles (NPs) on the energy policy of mammals. Thus, this study was undertaken to investigate the accumulation of Al_2O_3, CuO and TiO_2 NPs in the liver of female Wistar rats (*Rattus norvegicus* var. albinos) following oral administrations (0, 0.5, 5, 50 mg/kg b.w./day) for 14 days. Levels of total protein, lipid and glucose were measured to determine the energy reserves of the liver. ICP-MS (Inductively coupled plasma mass spectrometry) measurement showed that NPs accumulated in the liver, as the concentrations of Al, Cu and Ti increased sharply ($P<0.05$). Data showed that glucose levels decreased significantly ($P<0.05$) after NP exposures at all doses. Similarly, lipid levels also decreased ($P>0.05$) at the highest doses of Al_2O_3 and CuO exposures. However, total protein levels did not change ($P>0.05$) after any NP exposure. Likewise, the total energy reserves of the liver decreased ($P<0.05$) after the highest NP exposures. Interestingly, data indicated that the first energy sources (glucose and lipid) of the metabolism were decreased by all NPs, indicating possible metabolic stress.

Keywords: Metal, Nanoparticle, Rat, Liver, Accumulation, Energy.

1. Introduction

The release of metal-containing wastes to the environment has increased in recent years in parallel with the increase in human-induced activities. Mammals are exposed to metal-containing substances through the air, water and food. As a result of uncontrolled and untreated wastes of metal-containing products, many tragic accidents occurred including poisoning and death of animals and even humans [1]. One of the most famous of these incidents is the Minamata bay (Japan) disaster [2]. This event took place in the Minamata town in 1932, which caused the death or disability of dozens of people who ate fish contaminated with mercury. Another metal poisoning case occurred in Japan (around Jintsu River). In this case, people who ate the rice contaminated with cadmium developed "itai itai" disease which caused about 100 deaths [1]. Following the first large-scale metal poisoning events, the effects of metals on mammalian animals have been investigated by environmentalists, studying the accumulation patterns of possible toxic effects after metal exposures via different routes (e.g. oral, inhalation, intraperitoneal). In general, it has been shown that heavy metals can have mutagenic and carcinogenic effects as well as adverse effects on the nervous system, excretory system, circulatory system, reproductive system and osmoregulation of mammals [3].

With the advances in nanotechnology in recent years, metals have made a rapid entry into human life in the form of very small (<100 nm) matters called nanoparticles. Nanoparticles are used in many different fields such as the pharmaceutical industry, textile industry, filters, toothpaste, sunscreen, children's toys, humidifier, packaging products, white goods, electronic devices and food industry, and their application areas are increasing day by day [4]. Despite the fact that nanoparticles facilitate human life, reduce

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production costs and have widespread usage areas, there are not enough information about their environmental fates [5,6]. Studies carried out in different laboratories showed that nanoparticles could have toxic effects on different systems of different organisms starting from bacteria to humans [7]. Studies also demonstrated that the toxic effects of NPs vary considerably according to their chemical and physical properties, the dose applied, the route of administration and the affected organs and systems [8].

Nutrients such as carbohydrates, lipids and proteins are used as energy sources in animal cells depending on the level of energy requirements by the metabolism. In general, carbohydrates (as glucose) are used as immediate energy sources in animal metabolisms. However, lipids are used as a second energy source to fulfill the higher energy requirements, especially after a shortage of glucose in the serum. Nevertheless, proteins are the last employed molecules for this purpose, as they are mostly structural molecules or participate in vital molecules such as enzymes and hormones. Animals use high amounts of ATP under stressful conditions as their metabolic activities increase. It has been shown that organisms that lived under metal stress spend large amounts of metabolic energy to gain tolerance to metal toxicity [9]. Literature data demonstrated that metal stress changed the energy reserves of animals in relation to metal types and exposure conditions [10,11,12,13]. Therefore, xenobiotic exposures may be costly for animals in terms of metabolic activity and energy reserves, suggesting to use the energy reserves of animals as a biomarker to detect the stress caused by metals or metal-containing products.

Nanoparticles tested in the present study are used in different areas of industry such as the medical, electronics, chemical industry, biomedicine, military, cosmetics and food sectors [7,9]. Similar to the other xenobiotic, NPs are taken up by mammalians via different uptake routes (e.g. inhalation, food, water). The liver, one of the most metabolically active organs is the energy store of mammals and used to test the energy allocation under chemical stress [14]. Thus, the aim of the present study was to investigate the effects of Al_2O_3, CuO and TiO_2 NPs on the levels of nutrients and energy reserves in the liver of female Wistar rats after 14 days of oral administrations of NPs.

2. Materials and Methods

2.1. Experimental protocol

Female Wistar Albino rats (*Rattus norvegicus* var. albinos) used as test animals were obtained from Çukurova University Faculty of Medicine Experimental Medicine Research and Application Center (ÇÜTF-DETAUM). Since all the experiments were carried out in that laboratory, the rats were not taken out of the laboratory in which they were reproduced. Rats were randomly allocated into the experimental cages, each group containing 6 rats (a total of 60 female rats was used). Adult rats weighing 190-230 grams were used in the experiments and there was no significant difference among the exposure groups and controls ($P>0.05$). The room conditions in which the rats are kept were as follows; 12 hours of light: 12 hours of dark period, 50% humidity, and 22±1.0°C temperature.

NPs were purchased from Sigma Company (Germany). Stock solutions for each NP were prepared after sonicating vigorously (Bandelin HD2200 sonicator) for 20 min on the ice. Dilutions were done appropriately for the desired concentrations and mixed well before administration to rats. Prior to experiments, sublethal doses of NPs were determined and a series of sublethal NP doses (0, 0.5, 5, 50 mg/kg b.w./day) were daily administrated to rats via oral gavage. Rats were fed with standard rat food and allowed unlimited water. After 14 days, all rats were killed with high doses of anesthesia (ketasol 10%, Harson Lab. India) and dissected carefully using sterile equipment. Liver tissues were put in disposable tubes and stored at -80 °C (Esco UUS-480A) until the analysis. Experiments were done with the permission of the ethical Committee of Çukurova University (Code No:06.27.07.15).

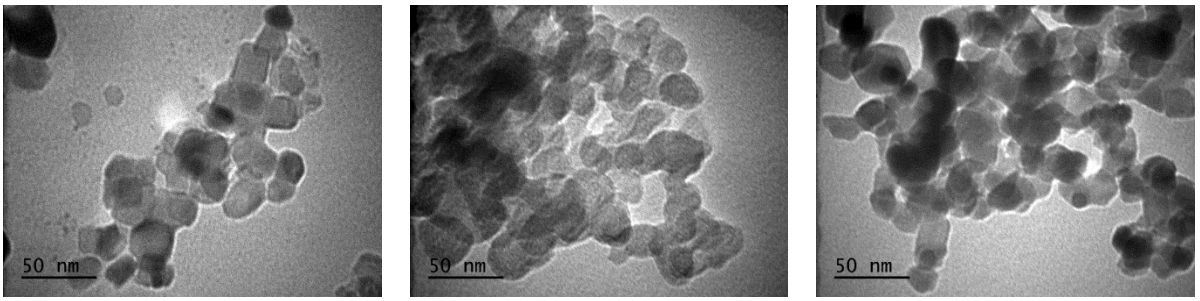
2.2. Measurements of nutrients and energy calculation

Measurements of glucose, lipid and protein were done as described in Emre et al. [15]. In brief, to measure the total glucose levels, a sample of the liver (approx. 200 mg) was homogenized for 5 min in 10% trichloroacetic acid (TCA) at 24,000 rpm and the homogenate was centrifuged to obtain the pellet. Then, the pellet was dissolved in ethyl alcohol and kept at 90 °C for 24 h. After that, this mixture was centrifuged for 30 min at 3,500 g to obtain the supernatants. Alcohol in the supernatant was evaporated in an oven at 37 °C. The contents of samples were analyzed by the Anthrone method of Plummer [16] and absorbance was read at 620 nm, using glucose as standard. To measure the total lipid contents of the liver, the same supernatants were used following the method developed by Van Handel [17]. After the evaporation of alcohol from the supernatant, a mixture of sulfuric acid and vanillin-phosphoric acid was added onto samples and absorbance was read at 525 nm. The corn oil (Sigma) was used as standard.

Total protein levels in the liver were measured by the method of Lowry et al. [18], using bovine serum albumin as a standard. The energy reserves of the animals are derived from energy supplying molecules such as carbohydrates, lipids and proteins. Each organic molecule has different (17.5 Joul/mg glycogen, 24.0 Joul/mg protein, 39.5 Joul/mg lipid) energy equivalents [19]. Details in calculations of energy reserves in organisms were given in our previous papers [12]. Briefly, the total energy reserve in the liver of an individual was calculated by summing the energy contributions of glucose, protein and lipid which were obtained by multiplying the energy equivalent value of each mg organic molecule.

2.3. Nanoparticle characterization

Characterizations of NPs are as follows; Al_2O_3 NPs have a size of ~40 nm and >30 m^2/g surface area, whereas CuO NPs have a size of ~40 nm and >20 m^2/g surface area and TiO_2 NPs have a size of ~21 nm and >30 m^2/g surface area. X-ray diffraction analysis (XRD) showed that gamma Al_2O_3 NP was polycrystalline structure and had a cubic phase, whereas Cu NP was polycrystalline structure and monoclinic phase and anatase TiO_2 NP was polycrystalline structure and tetragonal phase. Energy-dispersive X-ray (EDX) analysis was done using a field-emission scanning electron microscope (Zeiss/Supra 55 VP) to determine the contents of NPs. Data showed that Al, Cu and Ti contents (weight percentages) of Al_2O_3, CuO and TiO_2 NPs were 51.10%, 79.06% and 60.49%, respectively, the remaining atoms being only oxygen. Transmission electron Microscope (TEM) images of NPs were obtained using a Jeol JEM-1010 TEM (80 kW) connected to a GATAN 782 ES500W Erlangshen camera.



a) b) c)

Figure 1. TEM images of Al_2O_3 (a), CuO (b) and TiO_2 (c) nanoparticles in stock solutions [31]

2.4. Metal analysis

The levels of metals in the liver were measured by means of standard ICP-MS methods using the method published earlier [20]. Briefly, samples of liver tissues were put on Petri dishes to dry until constant weights in an oven (Nuve EN400). Then, dried tissues were weighed and put into the digesting flasks and 10 ml nitric acid (HNO_3) (65%) was added. Then, they were digested on a thermostated hotplate at 120 °C for 3 h. After complete digestion, samples were cooled and transferred to a microwave system (Berghof Speedwave XPRT) to digest further to make sure complete breakdown of NPs. Then, the digested samples were cooled and concentrations of Al, Cu and Ti were measured with an ICP-MS (Perkin Elmer, Nexion 2000p) which has detention limits (µg/L) of 0.00001, 0.00003 and 0.00003 for Al, Cu and Ti, respectively. For each metal, a series of standards were prepared using the stock standards. Standard solutions and appropriate reference material supplied from the manufacturer of the instrument (Perkin Elmer) were used to validate the measurements. Metal analyses were done in the Central Laboratory of Çukurova University.

3. Results and Discussion

There was only 5% rat mortality following gavage administration of NPs during the 14 days of the exposure period. Likewise, rats did not show any appetite loss and abnormal behavior during the experiments. Oral administration of Al_2O_3, CuO and TiO_2 NPs caused significant increases in the levels of Al, Cu and Ti in the liver, indicating accumulations of NPs (Fig. 2). Data showed that all metals were present in the liver of control rats and concentrations increased linearly depending on increases in administration doses. The highest concentrations (µg/g dry weight) of Al (46.2), Cu (43.6) and Ti (28.2) were measured at the highest exposure dose. The mean values and associated standard errors of glucose, lipid and protein in the liver of rats were presented in Figs 3-5. The mean total glucose level in controls was 11.2±0.30 mg/g w.w. After NP exposures, total glucose levels were decreased signifi-

cantly except the lowest dose of Al_2O_3 and TiO_2 (P>0.05). The mean total lipid level in controls was 3.20±0.19 mg/g w.w. Although the lipid levels decreased at all exposure conditions, significant (P<0.05) decreases occurred only at the highest dose of Al_2O_3 and CuO. The mean total protein level in controls was 47.6±0.95 mg/g w.w. Statistical analysis showed that protein levels in the liver of rats did not change significantly at any exposure condition (P>0.05). Energy equivalence values of glucose, lipid and protein were calculated as described in Canli [12] and presented in Fig. 6. According to this figure, the mean energy values (J/g w.w.) in the liver of controls was 1464±58.7. Total energy levels decreased in all NP exposed groups, though these decreases were not statistically significant (P>0.05) except the highest doses of Al_2O_3 and CuO exposures (P<0.05). Interestingly, statistical results changed dramatically when protein values were not involved in the total energy reserve calculations.

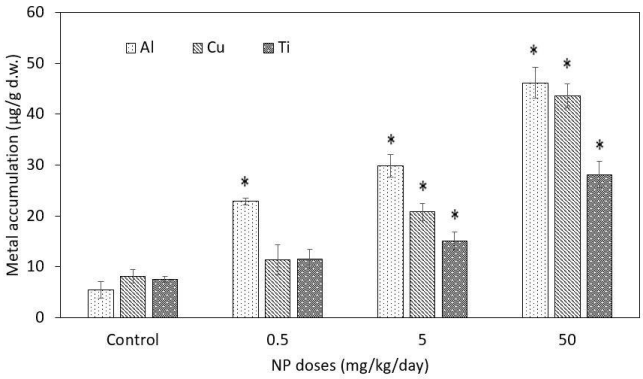


Figure 2. Accumulations of Al, Cu and Ti in the liver of rats after 14 days of oral administrations of Al_2O_3, CuO and TiO_2 NPs. * indicates significant (P<0.05) differences compared to controls (N=6)

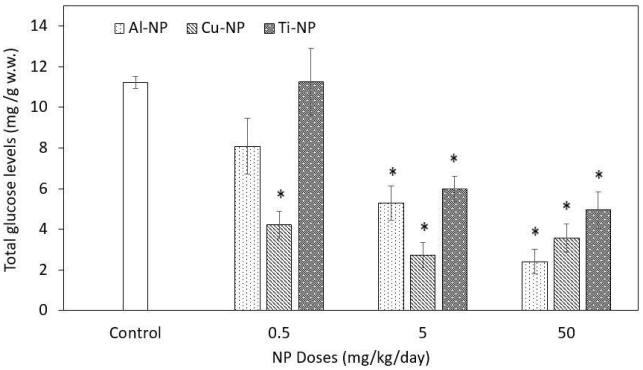


Figure 3. Glucose levels in the liver of rats after 14 days of oral administrations of Al_2O_3, CuO and TiO_2 NPs. *indicates significant (P<0.05) differences compared to controls (N=6)

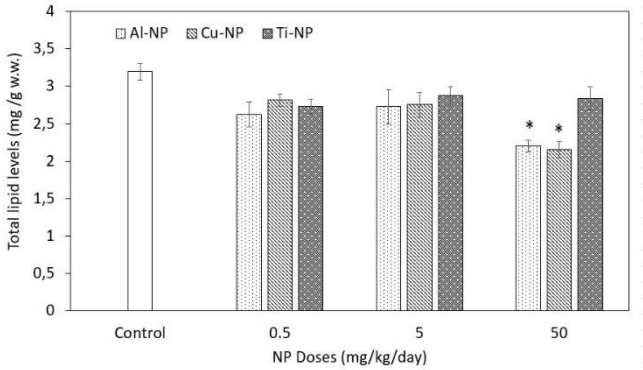


Figure 4. Lipid levels in the liver of rats after 14 days of oral administrations of Al_2O_3, CuO and TiO_2 NPs. *indicates significant (P<0.05) differences compared to controls (N=6)

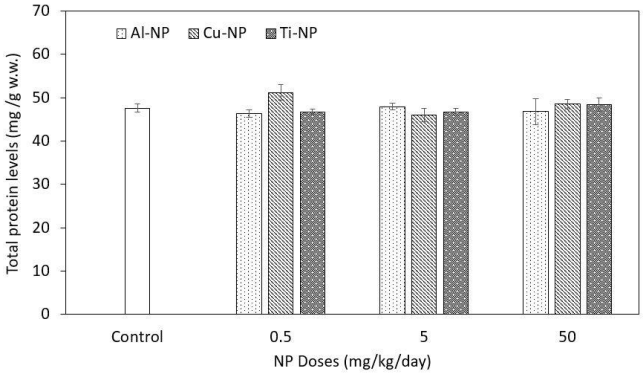


Figure 5. Protein levels in the liver of rats after 14 days of oral administrations of Al_2O_3, CuO and TiO_2 NPs. *indicates significant (P<0.05) differences compared to controls (N=6)

This study demonstrated that NPs were absorbed by the intestinal walls and entered into the blood and finally accumulated in the liver. The accumulation capacity of NPs was also noted by other researchers, indicating their potential toxic effects [6,21]. We also demonstrated the accumulation of Al_2O_3, CuO and TiO_2 NPs in tissues of rats, fish and mussels by means of TEM images [22,23,24]. Although literature data indicate that metal-oxide NPs have lower toxic effects compared to their dissolved metal forms, it is clear that they still seem to induce significant alterations in the biochemistry of mammals and other animals [5,6]. It is important to note that the physical characteristics of NPs (e.g. size) are very important in their tissue accumulation and subsequent toxicity [4,25]. Likewise, routes of administration (oral, inhalation subcutaneous, vein injection) were also found to affect in accumulation of NPs [21,26]. Thus, accumulation behaviours and toxic effects of NPs vary with size, shape, administration routes and physiology of animals in concern. As it is well known, animal metabolism first employs glucose as an immediate energy source, then lipids, and finally proteins. In this context, the immediate energy reserves of rats exposed to NPs significantly decreased when only glucose and lipid values were involved in the calculations. This may suggest that rats had shortages in immediate energy supply, possibly causing some disturbances in different metabolic systems.

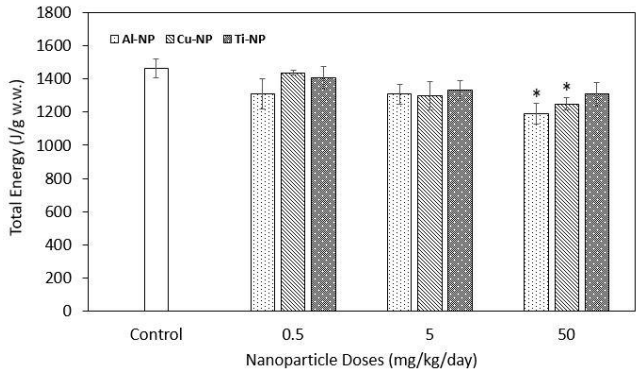


Figure 6. Total energy levels in the liver of rats after 14 days of oral administrations of Al_2O_3, CuO and TiO_2 NPs. *indicates significant (P<0.05) differences compared to controls (N=6)

The energy budget is an indicator of an organism's overall condition. Changes in energy reserves and/or energy consumption have been used as biomarkers of toxic stress [24]. Literature data demonstrated that the energy storage capacity of animals can alter following metal stress, the severity of alterations depending on metal type, exposure concentration and duration [10,11,12,15]. Therefore, the energy policy of animals could be a good biomarker to investigate metabolic stress caused by metals or metal-containing products. Świątek and Bednarska [24] studied the energy reserves (carbohydrates, lipids and proteins) in the earthworm (*Eisenia andrei*) after exposure to ionic and NP forms of zinc for 21 days and found that only carbohydrate levels significantly decreased, though this decrease did not affect the total energy budgets of animals. As occurred in the present study, total energy budgets do not change significantly when carbohydrates, lipids and proteins are involved in the calculation, because total protein levels do not generally change after exposure. However, the calculations without protein values demonstrate that the immediate

energy budgets of animals dramatically decrease, suggesting the metabolic stress points of view. Yeung et al. [25] also pointed the importance of the energy budgets of animals in environmental monitoring in an experiment carried out with the green-lipped mussel (*Perna viridis*). They indicated that not the carbohydrate levels but lipid levels in mussel tissues decreased after exposure to cadmium or copper. Canli [10] studied the energy reserves of carps (*Cyprinus carpio*) after exposure to mercury, chromium and nickel and found that energy sources of fish decreased sharply, mercury causing the highest decreases. Mammals also show similar trends in energy metabolisms when they are exposed to metals. Zhang et al. [26] studied the effects of subchronic exposure to low doses of Cd (10 µg/L) on the energy metabolism of mice. Data showed that exposure to Cd supplied in drinking water for 10 weeks increased hepatic triacylglycerol and serum fatty acid, indicating the dysregulation of energy metabolism due to Cd exposures. Likewise, He et al. [13] exposed mice to low cadmium concentrations in a chronic duration and found that Cd exposure induced the perturbation of energy metabolism in mice, evidenced by the alteration of various metabolites associated with the phosphorogen system, tricarboxylic acid cycle, and lipid metabolism. Ferreira et al. [27] exposed Wistar rats to different sizes (10 and 30 nm) of gold NPs (70 µg/kg/day) for 28 days via intraperitoneal administrations. They demonstrated that chronic gold NPs (10 nm) administration increased energy metabolism in the liver and decreased energy metabolism in the kidney and heart, whereas chronic gold NPs (30 nm) administration increased energy metabolism in the heart, indicating gold NPs can lead to oxidative damage and alterations in energy metabolism. We also demonstrated the effects of Al_2O_3, CuO and TiO_2 NPs in serum values in female Wistar rats including glucose, triglyceride and protein [28]. Data revealed that oral NP administration decreased glucose and triglyceride values in the serum, but not protein levels indicating the similarity with the present data. Yan et al. [29] studied the effects of ZnO NPs (100, 300 and 1000 mg/kg, respectively) in the urine and kidney of rats after exposing them to NPs for 14 days. Their findings showed that ZnO NPs disturbed energy metabolism and caused mitochondria and cell membrane impairments. Chen et al. [30] studied the effects of TiO_2 NPs orally administered to rats up to 90 days and found that NPs caused a slight and temporary hypoglycemic effect after 30 days of exposure, though there was some recovery after 90 days, suggesting the dietary intake of TiO_2 NPs as food additives could affect the absorption and metabolism of glucose. Although the authors could not determine NP presence in rats, our previous paper containing TEM images [31] and the present data clearly demonstrated that NPs including TiO_2 were accumulated by the liver of rats.

4. Conclusions

This study indicated that NP stresses were evident as the levels of the first energy supplies (glucose and lipid) of the rat metabolisms decreased following oral administrations of NPs. However, the levels of the last energy supply (protein) were not affected by NP administrations. Consequently, the energy reserves of the liver decreased but only at the highest doses of Al_2O_3 and CuO. Nevertheless, energy reserve calculations without protein values dramatically decreased at all NP exposures, suggesting the animals actually suffered from energy deficiency. Results also suggested that the effects of metal-oxide NPs should be investigated further to estimate better the consequences of NP exposures in different groups of animals.

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