

Histopathological Evaluation of Kidney and Heart Tissues after Exposure to Copper Oxide Nanoparticles in *Mus musculus*

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ABSTRACT

BACKGROUND AND OBJECTIVE: Copper nanoparticles are being extensively used in medical sciences, food supplements and industrial fields. However, their potential toxic effects on human health and the environment remain undetermined. The purpose of this study was to evaluate the toxic effects of copper oxide nanoparticles on kidney and heart tissues of mice.

METHODS: In this experimental study, 42 adult female mice, weighing 30±3 g, were randomly divided into control, sham and four experimental groups. The mice in the experimental groups intraperitoneally received copper oxide nanoparticles with doses of 300, 400, 500 and 600 mg/kg. After autopsy, the hearts and kidneys of mice were separated and weighed. For histopathological examinations, heart and kidney tissues were stained with hematoxylin and eosin.

FINDINGS: Kidney weight in control and sham groups, compared to experimental groups receiving nanoparticle doses of 300, 400, 500 and 600 mg/kg, reduced to 21±0.02, 19±0.02, 20±0.02 and 22±0.01 g, respectively, while no significant changes were observed in the heart weight. Histopathological examination of kidney and heart after the intraperitoneal injection of copper oxide nanoparticles showed signs of cytotoxicity including congestion, necrosis and inflammatory cell infiltration, compared to the control group.

CONCLUSION: The findings of this study showed that copper oxide nanoparticles cause damage to the kidney and heart in a dose-dependent way.

KEY WORDS: Copper Oxide Nanoparticles, Cytotoxicity, *Mus musculus*, Histopathology, Necrosis, Inflammatory Cell Infiltration.

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Introduction

Based on recent advances in nanotechnology, metal oxide nanoparticles can be used in various fields such as catalyzation, optical and electronic materials, sensors, environmental sanitation and biomedical sciences (1). Nanoparticles with a size range of 1-100 nm have particular physical and chemical characteristics in terms of size, shape, high surface area to volume ratio, solubility, activity and cell membrane penetration (2).

Sometimes, these nanoparticles are as small as cellular structures, viruses, proteins or genes. Although nanoparticles have many advantages, they can also lead to potential side-effects. Therefore, safety issues and the associated risks should be considered. These particles are even smaller than ordinary pollens and allergens and can cause hypersensitivity reactions (3).

Some nanoparticles may simply penetrate into sensitive lung tissues via breathing and cause damages, which can lead to chronic respiratory problems (4, 5). Therefore, the toxicity of nanoparticles should be noted since inhaling these compounds could be associated with responses such as chronic inflammation and production of free oxygen radicals (3). In some studies, some nanoparticles have been shown to cause fibrosis, inflammation and tumors (3, 6).

Nanoparticles have the ability to enter, move within and damage living organisms (7). This ability is mostly due to their small size, which allows them to cross physiological barriers and flow in the host circulatory system (8). Some particles, similar to viruses, can penetrate the lungs or skin barriers and enter blood circulatory and lymphatic systems in humans and animals; they can potentially disrupt cellular processes and cause diseases (9, 10).

Copper, following zinc and iron, is the third most abundant trace element in the human body. This element is a highly effective cation in electron-transferring reactions and binding with organic molecules. Copper is essential for cellular respiration, regulation of neurotransmitters, collagen synthesis and metabolism of nutrients, particularly iron. It also acts as an antioxidant against free radicals.

Copper exists in all living cells and is mostly a component of copper-containing enzymes and copper proteins. Copper-containing enzymes such as lysyl oxidase are essential for collagen and elastin

synthesis in connective tissues (11). Among nanoparticles, copper oxide has been applied in various industries, with negative impacts on the survival and growth of living organisms (12). Toxicity assessment of copper oxide nanoparticles has shown that its tracheal injection at high doses leads to acute inflammatory changes. It also induces chronic changes in the lung of rats, following its continuous injection at low doses (13).

The toxicity of copper oxide nanoparticles is associated with its solubility, which is dependent on temperature and pH of the solution. Copper oxide nanoparticles at a pH of 9-11 have trivial effects on solubility at room temperature and show minimal toxicity, while changes in pH unit severely increase its toxicity (14).

Nanoparticles cause toxicity via reactive oxygen species (ROS) production and induction of oxidative stress; this is in fact the most important mechanism of toxicity induced by nanoparticles. In living organisms, copper is one of the essential elements for maintaining homeostasis. Copper ions may cause toxicity as they exceed the physiological tolerance in living organisms. Consequently, the potential toxicity of copper oxide nanoparticles and the associated impacts on human health have greatly concerned the public and researchers (15, 16).

Studies on the effects of nanoparticles on human tissues are scarce due to differences in the distribution, influence, associated tissue damages and size of nanoparticles. According to literature review, it seems that no study has been conducted regarding the effects of nanoparticles (size=20 nm) on the heart and kidney of *Mus musculus*. Therefore, the purpose of this study was to evaluate the toxic effects of copper oxide nanoparticles on kidney and heart tissues of mice.

Methods

This experimental study was conducted on *Mus musculus*, purchased from Pasteur Institute of Iran (Amol, Iran). The animals were transferred and maintained in animal houses. Copper oxide nanoparticles were purchased from Nanosany Company (Mashhad, Iran) in form of black powder with 20 nm particles (99% purity, specific surface area of 25 m²/g and true density of 6.4 g/m³). In this experiment, adult male and female rats, with a weight range of 30±3 g, were used.

The animals were kept in standard conditions (12 hours of light and 12 hours of darkness, temperature of 23 ± 2 °C), with free access to food and water. Overall, 42 female mice with the mean age of 7-8 weeks were selected for the initiation of the main stages of the experiment. The animals were randomly divided into six groups (seven mice per group): control group, sham group and four experimental groups.

The required amount of the powder was mixed within a vial with a certain amount of sterile distilled water (double distillation). Then, the vial was vortexed for a few minutes until the content was well resolved. Afterwards, the black solution was pulled out with an insulin syringe and injected to animals. To determine the amount of copper oxide nanoparticles, LD₅₀ needed to be determined at the beginning of the experiment, given the fact that minimum lethal injection must be considered for animals (17).

According to pilot studies conducted before the experiment and dose-response studies, LD₅₀ for copper oxide nanoparticles (size=20 nm) was calculated to be 1000 mg/kg.

In this experiment, doses of 300, 400, 500 and 600 mg/kg were used. The experimental groups received copper oxide nanoparticles with doses of 300, 400, 500 and 600 mg/kg (volume of 0.4 ml) via insulin syringes. The sham group was injected 0.4 ml of distilled water.

Histopathological examinations:

For histopathological evaluations, the mice were sacrificed by cervical dislocation, and then autopsy was performed. Afterwards, the heart and kidney were removed from the body, and before fixation, their weights were measured, using a digital scale. For evaluating the histopathological changes, heart and kidneys were fixed in glasses, containing Bouin's solution.

Afterwards, tissue processing was performed, paraffin blocks were prepared and 5-micron sections were cut, using a microtome (Leitz 1512, Germany). The samples were stained by hematoxylin and eosin (H&E) and microscopic and histopathological studies were performed on tissue samples.

Statistical analysis: For data analysis, post-hoc Duncan's test and ANOVA were performed, using SPSS version 16. P-value less than 0.05 was considered statistically significant.

Results

Examination of the results regarding the effects of copper oxide nanoparticles (size=20 nm), which were intraperitoneally injected in *Mus musculus*, showed that changes in the mean heart weight in control, sham and experimental groups were not significant. The mean heart weight in experimental groups, receiving doses of 300, 400 and 500, was 0.26 ± 0.09 , 0.26 ± 0.04 and 0.25 ± 0.05 , respectively, which showed an increase, compared to the control group (table 1).

Table 1. Comparison of the mean weight of heart and kidney in *Mus musculus* after receiving different doses of copper oxide nanoparticles

Weight (g) Groups	Heart	Kidney
Control	0.23 ± 0.04 a	0.24 ± 0.01 a
Sham	0.24 ± 0.04 a	0.23 ± 0.02 a
Experimental (300 mg/kg)	0.26 ± 0.09 a	0.21 ± 0.02 a
Experimental (400 mg/kg)	0.26 ± 0.04 a	0.19 ± 0.02 a
Experimental (500 mg/kg)	0.25 ± 0.05 a	0.20 ± 0.02 a
Experimental (600 mg/kg)	0.21 ± 0.05 a	0.22 ± 0.01 a

The mean values with different alphabetical codes in each row are significantly different at 5% significance level in Duncan's test.

The mean amount of changes in kidney weight was significant in control and sham groups, compared to the experimental groups. The mean kidney weight in experimental groups, receiving doses of 300, 400, 500 and 600 mg/kg, was 0.21 ± 0.02 , 0.19 ± 0.02 , 0.20 ± 0.02 and 0.22 ± 0.01 , respectively; a significant reduction was detected, compared to the control group ($p < 0.01$).

The pathological study of heart tissues in the control and sham groups showed no significant pathological changes. The pathological study of heart tissues in the experimental groups, receiving doses of 300 and 400 mg/kg (nanoparticles per kilogram of body weight), showed a range of pathological changes including congestion, necrosis and mild cell infiltration (fig 1 & table 2).

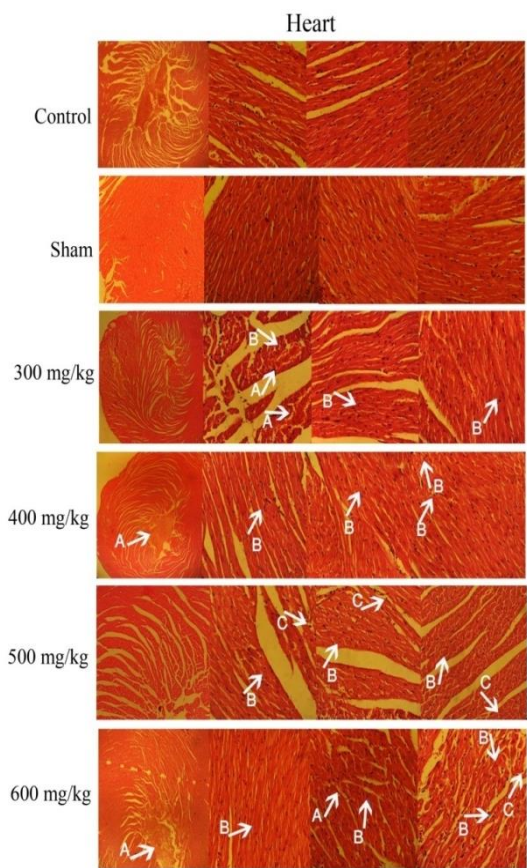


Figure 1. Cross-sections of heart tissues in *Mus musculus* after receiving different doses of copper oxide nanoparticles (H&E staining, 4× & 40× magnification); A: congestion, B: necrosis, C: infiltration of inflammatory cells

The pathological study of heart tissues in the experimental group, receiving 500 mg/kg of nanoparticles, revealed mild pathological changes, indicating congestion, while pathological changes were moderate regarding necrosis and inflammatory cell infiltration. At a dose of 600 mg/kg (copper oxide nanoparticles per kilogram of body weight), pathological changes of heart tissues became more significant by increasing the dose; in fact, tissue damages with higher intensity were observed (mild to moderate) (Figure 1 & Table 2). Histopathological study on *Mus musculus* showed that copper oxide nanoparticles at different doses could affect the kidney and cause congestion, necrosis, infiltration of inflammatory cells and vacuolar degeneration (Figure 2). However, in the sham and control groups, no significant pathological changes were observed. At a dose of 300 mg/kg, mild necrosis of kidney tissues was reported, whereas congestion and infiltration of inflammatory cells were mild.

Table 2. Comparison of the significance of histopathological changes in the heart of mice after receiving different doses of copper oxide nanoparticles

Groups Doses	necrosis	Congestion	Infiltration of inflammatory cells
Control	-	-	-
Sham	-	-	-
Experimental (300 mg/kg)	+	+	+
Experimental (400 mg/kg)	+	+	+
Experimental (500 mg/kg)	+	++	++
Experimental (600 mg/kg)	++	++	++

- Absence of tissue changes (natural structure), + mild damage, ++ moderate damage, +++ severe damage

At doses of 400 and 500 mg/kg, congestion, necrosis and infiltration of inflammatory cells were moderate, whereas vacuolar degeneration was mild. By increasing the dose of copper oxide nanoparticles, pathological changes in kidney tissues increased. In fact, at a dose of 600 mg/kg, in addition to mild congestion and infiltration of inflammatory cells, severe necrosis and other damages such as hyaline casts and vacuolar degeneration were reported (Table 3).

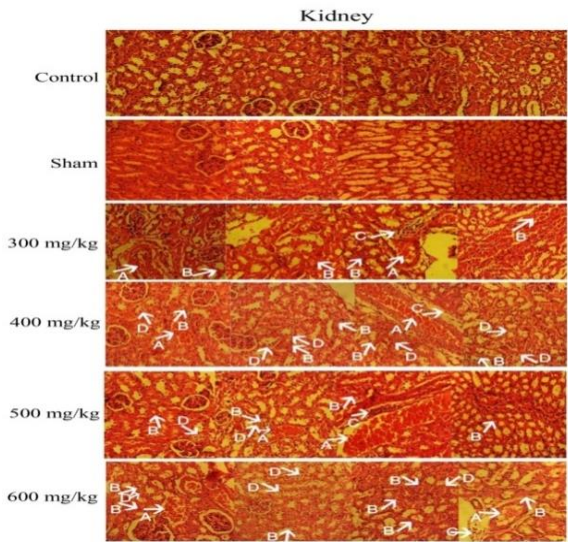


Figure 2. Cross-sections of the kidney of *Mus musculus* after receiving different doses of copper oxide nanoparticles (H&E staining, 4× & 40× magnification); A: congestion, B: necrosis, C: infiltration of inflammatory cells, D: vacuolar degeneration

Discussion

Histopathological study on the damages of heart and kidney tissues showed different ranges of tissue damage by increasing the dose of copper oxide nanoparticles. The results of histological studies showed that exposure to copper oxide nanoparticles with different doses could cause severe pathological changes in the heart such as congestion, necrosis and infiltration of inflammatory cells.

Moreover, at 300 and 400 mg/kg doses, blood vessels increased in volume, were filled with red blood cells and occupied a wide space. Also, congestion, necrosis and inflammatory cell infiltration were mildly observed in these groups, compared to the sham and control groups. By increasing the dose of nanoparticles, tissue damages increased (from mild to moderate), indicating the higher toxicity of copper oxide nanoparticles at higher doses, which eventually caused more severe tissue damages.

The size of nanoparticles is significantly associated with various characteristics such as the surface area, solubility and reactivity, which can affect the toxic behaviors of copper oxide nanoparticles in living organisms (18). Therefore, reduction in size leads to the expansion in the surface area of nanoparticles, which not only increases the congestion of nanoparticles, but also leads to increased reactivity and interaction between nanoparticles and biomolecules.

Our findings showed that copper oxide nanoparticles at higher doses inhibit cell proliferation by congestion. Also, the toxic effects result in congestion, infiltration and necrosis of heart tissues. In line with the present findings, Naghsh and colleagues examined the toxic effects of silver nanoparticles on heart tissues of Wistar rats and reported tissue changes and apoptosis (19).

The present study revealed that the mean heart weight increased in the experimental groups, compared to the control group, although these variations were not significant. The mean kidney weight declined in the experimental groups, compared to sham and control groups. These changes were indicative of the effects of copper oxide nanoparticles at different doses on kidney weight. In this regard, Lasagann-Reeves et al. showed that major nanoparticles were removed by the liver and spleen, following their injection and entrance to blood circulation. Heart and kidney tissues were in

the next places in terms of nanoparticle accumulation (20). In consistence with our findings, Liu et al. reported that the administration of copper oxide nanoparticles (size=25 nm) led to an increase in heart weight and a decline in kidney weight (21). The assessment of histopathological damages revealed that copper oxide nanoparticles in different doses cause various damages to kidney tissues. In fact, at different doses, congestion, necrosis, infiltration of inflammatory cells, hyaline casts and vacuolar degeneration were observed.

In this study, in all experimental groups (receiving different doses of copper oxide nanoparticles), mild congestion was observed in the kidneys, compared to the sham and control groups. Exposure to copper oxide nanoparticles led to tissue necrosis in a way that 300 mg/kg of nanoparticles caused mild necrosis. However, compared to the control group, by increasing the dosage, degeneration and necrosis were more observed in kidney tissues.

The histopathological damages of the kidney and the presence of vacuole-like structures, which were formed as holes, were indicative of vacuolar degeneration. At doses of 400 and 500 mg/kg, kidney damage seemed to be mild, compared to the control group. By increasing the dose, this type of damage became more critical in a way that at a dose of 600 mg/kg, more pathological changes were observed, which indicated the toxicity of copper oxide nanoparticles and dose-dependent damages.

In accordance with our findings, Chen et al. reported that copper oxide nanoparticles with different doses cause structural and pathobiological changes in the kidney. In addition, the toxicity of copper oxide nanoparticles in stomach and kidney tissues is caused by an increase in H⁺ and excessive production of $\cdot\text{HCO}_3$ (22). Therefore, in case of metal oxide nanoparticles, capillaries and lymph tissues play significant roles in absorption rate, and nanoparticles entering the blood vessels or lymph tissues may be transferred to other organs.

Meena et al. showed that nanoparticles cause cell apoptosis in kidneys via oxidative stress (23). Also, our study showed that at a dose of 600 mg/kg, in addition to the mentioned damages, cast hyaline (i.e., presence of protein compounds in the lumen or in the middle of urinary tract, as well as the absence of proper filtration and protein excretion) was observed in the urinary structure, which indicates the

incapability of the urinary system (glomerulus and urinary tract). Our findings showed that after two intraperitoneal injections of copper oxide nanoparticles with different doses, major histological changes were observed in heart and kidney tissues of mice. These findings showed that the flow of nanoparticles in various cell membranes results in their penetration into the blood stream and eventually the heart and kidney. In addition, changes in the weight of heart and kidney tissues, as well as tissue damages in these organs, indicated the toxic effects of copper oxide nanoparticles at different doses during the experiment.

The mechanism of toxicity in copper oxide nanoparticles is dependent on nanoparticle interactions with biomolecules, ROS production and oxidative stress induction. By increasing the production of ROS, nanoparticles can destruct DNA, increase the expression of death receptor genes, cause DNA point mutations, induce double- or

single-strand breaks in DNA and lead to impaired mitochondrial function and finally cell death (24-27). Also, in the present study, at higher doses, copper oxide nanoparticles led to the necrosis of kidney and liver tissues, compared to lower doses. This supports our findings regarding the necrosis of kidney tissues at higher concentrations.

The current histopathological studies showed considerable damages in kidney and heart tissues of mice, receiving copper oxide nanoparticles (size=20 nm). These results can provide the grounds for more comprehensive studies on different doses of nanoparticles at different time intervals.

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