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# Serum biochemical status and morphological changes in mice ovary associated with copper oxide nanoparticles after thiamine therapy

**Rahmat-Allah Fatahian-Dehkordi[1*$], Mahbobe Reaisi[2], Mohamad-Saeed Heidarnejad[3$], Abdonnaser Mohebbi[4]**

[1]Department of Basic Sciences, Faculty of Veterinary Medicine, University of Shahrekord, Iran
[2]Master of Animal Physiology, Faculty of Sciences, University of Shahrekord, Iran
[3]Department of Biology, Faculty of Sciences, University of Shahrekord, Iran
[4]Department of Clinical Sciences, Faculty of Veterinary Medicine, University of Shahrekord, Iran

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**ABSTRACT**

**Introduction:** Nanoparticles (NPs) can induce inflammatory responses and oxidative stress and are also cytotoxic to the genital organs of animals after exposure. The aim of this study was to assess the effects of copper oxide (CuO) and CuO NPs alone and in combination with thiamine on the ovaries of mice and on antioxidant enzymes.

**Methods:** Sixty adult mice were randomly divided into five groups. Group A served as the control. Group B received CuO NPs and group C received CuO at 0.2 mL/kg intraperitoneally (IP). Mice in groups D and E respectively received CuO and CuO NPs along with thiamine (30 mg/L) therapy. The responses of the ovaries to the treatments were appraised by histopathology studies. The values for catalase (CAT), superoxide dismutase (SOD), and lipid peroxidation were determined after 20 days of treatment.

**Results:** The degree of degeneration and apoptosis of the different zones within the ovaries were recorded in groups B and C. The decrease in CAT value and increase in SOD activity were significant for groups B and C at 20 days compared to the control group. The thiobarbituric acid reactive substances (TBARS) level in groups B, C and E were significantly higher at 20th day when compared with control group. The groups treated with thiamine showed histopathological and enzymatic results that were similar to those of the control group.

**Conclusion:** These findings suggest the combination of CuO NPs and CuO with thiamine improves serum enzyme activity and has positive effects on the ovary.

*Implication for health policy/practice/research/medical education:*
It seems that thiamine can somewhat control serum enzyme activity and ovarian tissue structure against destructive role of CuO NPs and CuO in mice.

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## Introduction

The application of nanoparticles (NPs) in daily life has increased and studies have assessed the biological effects of nano-composite materials and different types of NPs, particularly on the structure and function of organs. The toxicity of NPs and products related to them are important biological mechanisms that can endanger human health. These mechanisms and the effects of engineered and natural NPs depend on factors such as surface functionalization, aggregation, size, crystallinity, and composition (1,2).

The simplest copper (Cu) compound is copper oxide (CuO), which features physical properties such as electron correlation, high temperature superconductivity, and spin dynamics (2,3). CuO also has photoelectric properties related to its crystalloid structure and narrow band gap

*Corresponding author: Rahmat-Allah Fatahian Dehkordi, Department of Basic Sciences, Faculty of Veterinary Medicine, University of Shahrekord, Shahrekord, Iran. Email: fatahian_1349@yahoo.com
$Rahmat-Allah Fatahian Dehkordi and Mohammad Saeed Heydarnejad are considered as the first authors.

(4). Metal oxide NPs have attracted considerable attention because of their potential uses. CuO NPs are commonly utilized in conductive coatings, microelectronics, heat transfer fluid in machinery, and in lubricant additives (2). CuO NPs also possess antibacterial characteristics when exposed to *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The antibacterial activity of these NPs has been synthesized by thermal decomposition in vitro (5). Studies have shown that CuO NPs used in industrial applications can be released into the environment resulting in damage to and destruction of cells. CuO NPs scatter in the organs and tissues of animals and alter their structures (6). CuO NPs in a dose that approaches its threshold can cause tissue necrosis and dystrophy (1). The destructive effects of respired CuO NPs on the lungs of mice has been demonstrated (7).

Other destructive effects of NPs are inflammation, genetic damage, oxidative stress, inhibition of cell division, and cell death (1). Researches have shown that redox reactions in biological systems produce toxic reactive oxygen species (ROS) and related derivatives. Oxidative stress is the result of the generation of ROS such as hydroxyl radicals, organic hydroperoxides, hydrogen peroxide, and nitric oxide. Oxidative stress can produce lipid peroxidation, DNA injury, intra-cellular signaling networks related to carcinogenesis, a decrease in cell growth, and fibrosis (8). It has been shown that some NPs can activate inflammatory cells, macrophages, and neutrophils, which can increase the generation of ROS. Glutathione peroxidase (GSH-Pxs) enzyme, superoxide dismutase (SOD), and catalase (CAT) protect cells confronting damage caused by free radicals (9). Biological fluids and cells possess a pattern self-protective antioxidant behavior; thus, protocols have been developed to determine serum antioxidant activity. The most important protocols are based on reduced production of thiobarbituric acid reactive substances (TBARS); these have been used for rat liver homogenates (10).

Thiamine, vitamin B1, is a necessary part of any diet and have important role to fight off diseases for example nephropathy and beriberi, so its existence within body is required and have an important commitment. Thiamine is a water soluble nutrient and need for growth, evolvement and cellular function. Additionally considered as a ligand of the cell special for purposive surrender because thiamine possess a defined transport system within cells (11).

The current study examined the effects of IP injection of CuO NPs and CuO alone and in combination with thiamine on the ovaries tissue and biochemical activity of some enzymes in mice. Therefore, CuO NPs, CuO and thiamine with distinctive dosages were prepared and subsequently their effects evaluated.

## Materials and Methods

### Experimental animals and protocol

The investigation was managed on 60 pathogen-free female mice (strain of BALB/c) with weight 22-27 g. They was maintained in stainless steel wire cages in a proprietary animal room regulated at temperature of 22°C–23.5°C and relative moistness 32 %, with 14-hour light and 10-hour dark cycle. Animals' food including standard laboratory pellets and water both were available ad libitum. Total animals were adapted and quarantined for 14 days in a proprietary animal room. Animal choice, management and experiment protocol followed the routines approved by the Institutional Animal Care and Use Committee, Shahrekord University, Shahrekord, Iran.

### Copper oxide nanoparticles Preparation

The all chemical reagents used were purchased from Merck (Darmstadt, Germany) for this investigation. Reactivity were performed under atmosphere of air and in synthesis procedure, $CuSO_4.5H_2O$ was dissolved in deionized water. The solution was stirred by magnetic stirrer at 100°C. In pH 8, great amount of Cu(OH)2 precipitate was formed and then powders were toughened for 1.5 hours to achieve the crystalline CuO NPs.

### Grouping

After acclimatization, animals were allocated into 5 treatment groups with nearly equal mean body weights. Saline was intraperitoneal injected to the control group (group A). Group B mice received 0.2 mL/kg from CuO NPs IP, group C received 0.2 mL/kg BWs from CuO IP, group D received 0.2 mL/kg BWs from CuO NPs along with thiamine (30 mg/L) IP and group E received 0.2 mL/kg BWs from CuO along with thiamine (30 mg/L) IP. All experimental groups consisted of twelve mice in each group. Clinical symptoms were monitored throughout 20 days study period and gross results were observed on the scheduled necropsy day. During the study, all animals were observed once daily after treatment for health, mortality and any clinical symptoms of toxicity. Consumption of the food and water was checked daily after the start of any treatment.

### Blood collection and preparation

In the study, we used the serum of animals; on day 20, mice were sacrificed under anaesthetization in slight chloroform and bleeding was performed from their heart. The specimens were put in acid-washed polyethylene tubes without anticoagulation agent, and then allowed to clot.

### Thiobarbituric acid reactive substances

Initially, a blank sample including of 2 mL reagent and 1 mL distilled water were prepared. The reagent was prepared through the addition of trichloroacetic acid (10%) (w/v) and 0.375% thiobarbituric acid (TBA) (w/v) to 0.025 N HCl, and heated gently until TBA was completely dissolved. A serum sample of 0.5 mL was mixed with 0.5 mL sterile distilled water in a labeled 1.5 mL micro-centrifuge tube.

After the addition of 2 cc reagent to each sample, the

micro-tubes were immersed in 37°C water bath for 10 minutes, placed at room temperature for 20 minutes and centrifuged at 1500×g for 20 minutes. The absorbance values were measured at 535 nm, after the supernatants were transferred to new-labeled tubes. Also, the anti-oxidative activity was measured using malondialdehyde index (1.056×105 $M^{-1}$ $cm^{-1}$) (12).

### Superoxide dismutase assay

Enzyme activity was determined based on the method described by Sun et al (13). Activity method is based briefly on the inhibition of nitroblue tetrazolium (NBT) reduction by the xanthine-xanthine oxidase system as a superoxide generator. One unit of the SOD was defined as the enzyme activity causing 50% inhibition in the NBT reduction rate.

### Catalase assay

The enzymatic activity of the CAT was determined by spectrophotometry, following the hydrogen peroxide ($H_2O_2$) degradation rates for 1 minute, according to the method of Goth (14). Briefly, 0.1 mL of serum was added to 1 mL reaction mixture that included 50 mmol $L^{-1}$ potassium phosphate buffer (pH 7.0) and 10.6 mmol $L^{-1}$ $H_2O_2$. After 60 seconds, the addition of 0.5 mL of 32.4 mmol $L^{-1}$ ammonium molybdate solution inhibited the reaction with the formation of a yellow complex consisting of ammonium molybdate and hydrogen peroxide. The absorption spectrum of the substrate was calculated at 405 nm with a spectrophotometer versus a blank. One unit of the CAT activity was determined by the amount of enzyme decomposing 1.0 µmole of $H_2O_2$ per minute at pH 7.0 and 25°C.

### Histology preparations

The ovary organs from all animals were assembled on day 20 at necropsy and fixed in 10% buffered neutral formalin solution and were dehydrated with grades of ethanol (70% to 100%). Paraffin-embedded sections were cut at 5-6 µm and stained using hematoxylin and eosin. Samples visualized under light microscope (Olympus) to study the microscopic architecture of the ovary

### Statistical analysis

The findings were analyzed statistically using SPSS software and one-way analysis of variance (ANOVA) was performed to evaluate the significance of differences. Also, the Tukey multiple comparison tests were used for further evaluations at the level $P<0.05$.

## Results

The mice administrated with saline (group A) did not develop any clinical symptoms of toxicity either immediately post-treatment period. Histological examination of the ovaries collected from control mice showed no histopathological alterations. The ovary appeared normal and had follicles in different stages and corpora lutea. In treated ovaries by CuO NPs and CuO (groups B and C, respectively), the follicles showed degenerative alterations and did not show their normal shape and arrangement of granulosa cells, these events were more severe in group C when compared with group B following 20 days of treatment. Infiltration of the inflammatory cells such as lymphocytes had occurred around the blood vessels (perivascular infiltration); also, there were signs of degeneration in corpus luteum. In the CuO NPs and CuO groups along with thiamine (group D and E, respectively), histopathological alterations seen were somewhat close to normal, with group D appearing histologically closer to normal than group E. In the latter group (E), the therapeutic effect of thiamine was less evident and histopathological findings were approximately close to B and C groups.

### Catalase

The data presented for CAT activity in different groups are summarized in Table 1. The results showed that CAT activity significantly decreased in B and C groups treated with CuO NPs only (0.2 mL/kg BWs; $P<0.05$) and with CuO (0.2 mL/kg BWs; $P<0.05$) respectively, on 20th day. However, it was detected that the reduction in CAT activity of D and E groups was less and close to control group.

### Superoxide dismutase

Changes in amounts of SOD activity in experimental groups are given in Table 2. The SOD activity of serum was increased in CuO NPs and CuO groups ($P<0.05$) on 20th day than control group. Besides, there were no statistically significant increase in SOD activity of all samples between D and E groups than control group (the mean, 24.52, $P>0.05$).

### Thiobarbituric acid reactive substances

It should be noted that an increase had occurred on 20th day in the TBARS values (Table 3) of the all groups in comparison with control group. By comparing the means of the five groups together, it was detected that there were no significant differences in the TBARS values on 20th

**Table 1.** The changes of catalase activity during the different days of sampling

| Parameters | Days | G A (Control) | G B (CuO NPs) | G C (CuO) | G D (CuO NPs, Th) | G E (CuO, Th) |
|---|---|---|---|---|---|---|
| CAT (Umg $^{1}$ protein) | 20 | 172.82 ± 7.69 | 139.30 ± 17.56[a] | 130.34 ± 3.44[a] | 152.33 ± 15.56 | 149.15 ± 14.06 |
| *P* value | | >0.05 | <0.05 | <0.05 | >0.05 | >0.05 |

Abbreviations: Th: Thiamine; CuO, copper oxide; NPs, nanoparticles.
Means ± SD for data analysis.
[a] $P<0.05$, significant decrease to controls.

**Table 2.** The changes of superoxide dismutase activity during the different days of sampling

| Parameters | Days | G A (Control) | G B (CuO NPs) | G C (CuO) | G D (CuO NPs, Th) | G E (CuO, Th) |
|---|---|---|---|---|---|---|
| SOD (% inhibition) | 20 | 25.16 ± 3.17 | 40.66 ± 3.81[a] | 41.20 ± 3.88[a] | 38.26 ± 5.77 | 36.26 ± 1.18 |
| *P* value | | >0.05 | <0.05 | <0.05 | >0.05 | >0.05 |

Abbreviations: Th: thiamine; CuO, copper oxide; NPs, nanoparticles; SOD, superoxide dismutase.
Means ± SD for data analysis.
[a] *P*<0.05, significant increase to controls.

**Table 3.** The amount changes of TBARS during the different days of sampling

| Parameters | Days | G A (Control) | G B (CuO NPs) | G C (CuO) | G D (CuO NPs, Th) | G E (CuO, Th) |
|---|---|---|---|---|---|---|
| TBARS | 20 | 3.23 ± 0.04 | 5.19 ± 0.54[a] | 5.27 ± 0.35[a] | 3.59 ± 0.28 | 5.00 ± 0.35[a] |
| *P* value | | >0.05 | <0.05 | <0.05 | >0.05 | >0.05 |

Abbreviations: Th: thiamine; CuO, copper oxide; NPs, nanoparticles; TBARS, thiobarbituric acid reactive substances.
Means ± SD for data analysis.
[a] *P*<0.05, significant increase to control group.

day ($P>0.05$) in comparison with the control group.

## Discussion

In present study, two main topics were investigated. First, we wanted to determine if the CuO NPs and CuO concentrations differentially affect the activities of CAT, SOD and levels of TBARS in the serum of mice. Secondly, we wanted to determine that if in mice treated by CuO NPs and CuO supplemented with thiamine would result in activity changes of related enzymes as compared to mice without treatment (control) and mice with treatment only. It appears that the enzymatic activity of CAT decreased significantly, particularly in the groups receiving CuO NPs and CuO at 20 days after injection. The enzymatic activity of SOD increased significantly in both groups at 20th day.

Copper is a component of important enzymes involved in biological processes. Because Cu is found in the active units of necessary proteins, this metal can be released and break free to catalyze and generate reactive hydroxyl radicals, (15-17). A principal self-protective behavior against cyto- and geno-toxicities of nano-scale particles is oxidative stress (18). Metallic endogenous oxidative stress derived from ROS can injure bio-molecules such as DNA (19).

Among different metal oxide NPs, CuO NPs are used in magnetic, electrical, and thermal processes. Exposure to CuO NPs in high does can induce severe acute alteration; studies have revealed that CuO NPs cause damage to DNA and are cytotoxic to lung epithelial cells (20). It appears that the cytotoxicity effects of nano-materials, especially CuO NPs, include incremental generation of intra-cellular ROS. Fu found that oxidative stress is a major mechanisms of cytotoxicity of CuO NPs caused by the formation of ROS (21). Stambe et al showed that the anti-oxidant activity of ROS metabolizing enzymes such as CAT and SOD affects NPs-induced ROS (22). The present study used NPs to study biochemical changes and antioxidant activity and the responses of CAT and SOD to CuO NPs and CuO in the ovaries of mice.

At 20 days, group B recorded a decrease in CAT activity and group C recorded an increase in SOD activity when compared to results in the control group. The data suggests that continuous exposure of mice to CuO NPs and CuO for 20 days caused biochemical changes. In agreement with the results of the present study, a previous study found that rats with mammary carcinogenesis fed a diet containing copper showed a significant decrease in CAT activity in comparison with the control groups (23). Another study found no differences in CAT and SOD activity between the group injected with CuO NPs and the control group (24).

The results showed that CuO NPs and CuO can induce lipid peroxidation, The TBARS levels increased in groups B and C at 20 days in comparison with control group. The induction of oxidative stress by lipid peroxidation is a natural effect of CuO NPs. These findings are in line with those of previous studies which found oxidative stress originates from CuO and CuO NPs and produces geno-toxicity and lesions on oxidative DNA (25). Changes in TBARS indicated some defense against damage caused by oxidative stress in the groups that received CuO and CuO NPs.

Thiamine is a micro-nutrient that can be dissolved in water and is necessary for growth, the functioning of cells, and their development. Thiamine is considered to be a special ligand of cells for targeted delivery because total eukaryotic cells possess transmission mechanisms for thiamine (11,26). Thiamine in a diet containing bracken ferns was found to reduce bladder tumors in rats (27). The applications of thiamine in the form of NP coatings and the defensive effects of thiamine supplements have shown that thiamine has a protective effect (11,26,28). In the present study, CAT and SOD activity and levels of TBARS was affected by CuO and CuO NPs (Tables 1 to 3) and thiamine supplementation reversed many of these changes. The difference in the results could be associated with the protective effects of thiamine. This suggests that the administration of parenteral thiamine to mice injected with CuO and CuO NPs reversed their destructive effects.

Evidence of cytotoxicity have been reported in several in vitro studies of the cellular responses to CuO and CuO NPs. Researchers have shown that different results for CuO and CuO NPs toxicity depend on the manner of NP administration, cell type, and alternative routes of administration (29). The present study used nano-sized particles to determine the histological changes in the ovaries of mice. The results showed changes in the structure of the ovaries at 20 days after IP administration of CuO and CuO NPs. There was influx of lymphocytes around the small blood vessels by perivascular infiltration and degeneration in the graafian follicles. A study conducted on CuO NPs found that cellular degeneration affected the proteins and organelles of the cytoplasm. This degradation continued and led to cellular self-digestion (autophagy). Sun et al found that CuO NPs can cause cell degeneration through autophagic routes (13).

In conclusion, degenerative effects were found in cells of the ovaries of mice caused by oxidative stress after exposure to CuO and CuO NPs. The investigation showed changes in the antioxidant enzymes and microscopic findings decrease with administration of CuO and CuO NPs together with thiamine.

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## Authors' contributions

RF and MSH: designing study; RF: histological examination and statistical analysis; MR: performing animal experiment and histological examination; RF: manuscript preparation and its editing.

## Conflict of interests

None of the authors has any conflicts of interest to declare.

## Ethical considerations

Authors have been entirely regarded ethical issues such as: plagiarisms, data assembly, fraud, double publication or submission. Also all animal experiments were approved by the institutional ethical committee and carried out according to the International Guide for the Care and Use of Laboratory Animals.

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