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ORIGINAL ARTICLE

Histopathologic and biochemical assessment of mice ovarian tissue, following experimentally induced copper poisoning

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Abstract

In this study we investigated histopathological changes of mice ovarian tissues following long-term administration of copper sulfate and induction of chronic copper poisoning. The study comprised of three different groups of twenty one mice as follows: The first group (Group 100) which treated by gavage with copper sulfate at a dose of 100 mg kg^{-1} for 5 weeks. The second group (Group 200), which received 200 mg kg^{-1} copper sulfate by gavage during experimental period (35 days), and control animals (Group C), which received the same volume of distilled water in the same way. The blood samples were obtained from 7 cases out of 21 animals of each group by cardiac puncture at the end of first, third and fifth week. Ovarian lesions were observed in group 100 after 35th day and in group 200 after 21st day. The histopathologic examination revealed widespread ovarian follicle atresia in group 200 after 35th day Atretic follicles had numerous cell debris and inflammatory cells in the antral cavity. In degenerative antral follicles granulose cells degenerated and desquamated into antrum. In some degenerative antral follicles infiltration of inflammatory cells into granulose cell layer and antrum were observed. The histopathlogic data indicate the possibility of adverse effect of copper poisoning on the mice ovarian tissue. Copper might be mediator of the effect of oxidative damage and play an essential role in reproductive system.

Introduction

Copper is an essential trace element that is widely distributed in animal tissues. It is a component of a number of metalloenzymes such as catalase, peroxidases, and cytochrome oxidase, and is essential for the utilization of iron.[1,2] Copper sulfate is the most common copper salt, however, other important copper salts include carbonate, cyanide, oxide, and sulfide.[3] Copper can be absorbed into the systemic circulation from the gastrointestinal tract, lungs, and skin[3]. The gastrointestinal absorption of copper is influenced by a number of factors, including its chemical form: soluble copper compounds (oxides, hydroxides, citrates and sulfate) are readily absorbed but water-insoluble compounds (sulfides) are poorly absorbed.[4] Absorbed copper binds to plasma albumin and amino acids in the portal blood and is transported to the liver where it is incorporated into ceruloplasmin and later released into the plasma.[3]

The general population is exposed to metals at low concentrations either voluntarily through supplementation or involuntarily through intake of contaminated food and water or contact with contaminated soil, dust, or air. Contact with copper may also result from use of copper fungicides and algaecides. Workers may be exposed to copper in agriculture, and in various industries such as copper production and metal plating. The largest anthropogenic releases of copper to the environment result from mining operations, agriculture, solid waste, and sludge from sewage treatment plant, such as windblown dust and volcanic eruptions, may be significant.[3]

Although the chronic toxicity from long-term exposure to copper has not been investigated extensively, studies of patients such as Wilson's disease, a genetic defect that results in accumulation of copper in tissues, provide information on the chronic toxicity of copper.[3]

According to the authors' knowledge, there is not enough information about the copper toxicity on histopathological feature of mice ovaries. Therefore, the aim of the current study was to investigate histopathological changes of mice ovarian tissues following long-term administration of copper sulfate and induction of chronic copper poisoning.

Materials and Methods

Animals. Sixty-three sexually matured female NMRI mice were purchased from the Animal Laboratory of Kerman University of Medical Sciences (KUMS), Kerman, Iran and kept in the Center for Laboratory Animal Care at the School of Veterinary Medicine of Shahid Bahonar University of Kerman for 1 week before treatment. The mice weighed 25–30 g and were the same age (1.5 – 2 months old). The experimental animals were randomly divided into three groups of twenty-one animals each and were housed in standard polypropylene cages with wire mesh top, at 21°C in a 12 h/12 h dark-light cycle. During the study, the animals received water and pellet food (Javaneh Khorasan Co, Iran) *ad libitum* plus vitamins and minerals premix containing vitamin A, D_3, E, K, B_1, B_2, Ca-pantothenate, Nicotinic acid, Pyridoxine, Folic acid, Biotin, Cholic acid, Mn, Mg, Zn, Fe, Cu, Co, I and Se. All ethical considerations using animals were considered carefully.

Experimental design. The study comprised of three different groups of twenty one mice as follows: The first group (Group 100) which treated by gavage with copper sulfate at a dose of 100 mg kg^{-1} for 5 weeks. The second group (Group 200) which received 200 mg kg^{-1} copper sulfate by gavage during experimental period (5 weeks), and control animals (Group C), which received the same volume of distilled water by gavage during experimental period.

Blood sampling. The mice were euthanized by intra-cardiac injection of sodium thiopental and blood samples were obtained from 7 cases out of 21 animals of each group by cardiac puncture during experimental period at the end of first, third and fifth week. Whole blood was collected aseptically using sterile 2 mL syringe and poured into tubes without anticoagulant. The blood was centrifuged (Shimifan, Iran) at 3000 g for 10 minutes at room temperature, and sera were harvested using disposable pipettes and transferred into 1.5 mL sterile micro tubes (Eppendorf, Germany). Serum samples were kept at −20 °C until the day of analysis.

Biochemical parameters. The serum concentration of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were determined by biochemical automatic analyzer (Autolab®, AMS®; Rome, Italy), using commercial kits (Pars Azmoon, Iran). Serum and hepatic copper concentration were analyzed by an atomic absorption spectrometer (Buck Scientific Co., USA).

Histopathological assays. After necropsy, the liver and ovary samples from 7 cases out of 21 animals of each group were preserved in 10% neutral buffered formalin solution for histological examination at the end of first, third and fifth week. Formalin-fixed samples were processed by the standard paraffin wax technique, and sections of 5 µm thickness were cut and stained with hematoxyline and eosin (H&E).

Statistical analysis. All data were expressed as mean ± standard error (SE) of the mean. Statistical analysis was performed using one-way analysis of variance (ANOVA), followed by Post Hoc, Bonferroni test. A value of $P < 0.05$ was considered statistically significant.

Results

Results of serum biochemical parameters are presented in Tables 1 and 2. Analysis of biochemical data revealed that serum levels of AST and ALT in group 200 after 3rd week of experimental period were significantly increased ($P < 0.05$) in comparison with those of group C.

The data obtained from serum and hepatic samples analysis are presented in Table 1 and 2. The results showed that serum level of copper in group 100 after one week and in group 200 after 21st day of experimental period was significantly increased ($P < 0.05$) in comparison with that of group C. The liver copper concentration was gradually increased and after 21st day of experimental period was significantly increased ($P < 0.05$) in comparison with that of group C.

In the control group, liver had normal hepatic lobules and hepatocytes without any histopathologic lesions. Hepatic lesions were observed in group 100 after 35th day and in group 200 after 21st day of experimental period and then became more severe during the experimental period. The histopathologic examination revealed hepatocellular degeneration and necrosis. Multifocal infiltration of lymphocytes and plasma cells were observed (Fig. 1). Central veins were dilated and filled with erythrocytes. Other hepatic lesions included: bile pigment retention in hepatocytes and biliary canaliculi.

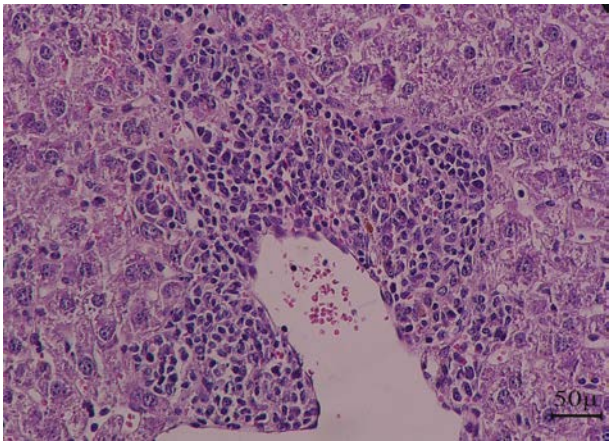

Fig 1. Liver. Group 200 after 35th day. Infiltration of lymphocytes and plasma cells in periportal area. H&E. (400×)

Table 1. Levels of serum AST, ALT and copper and liver copper concentration following administration of copper sulfate at a dose of 100 mg kg^{-1} per day for 35 days.

Parameters	Control group	Group 100		
		7th day	21st day	35th day
AST (IU L^{-1})	75.40 ± 1.25 a	77.14 ± 0.25 a	77.40 ± 0.36 a	77.80 ± 0.34 a
ALT (IU L^{-1})	159.00 ± 1.61 a	161.1 ± 0.50 a	163.8 ± 0.91 a	168. 30 ± 0.71 b
Serum Cu (µmol L^{-1})	14.30 ± 0.18 a	17.07 ± 0.49 b	19.85 ± 0.67 c	21.57 ± 0.65 c
Liver Cu (mmol kg^{-1})	4.65 ± 0.06 a	4.64 ± 0.12 a	7.67 ± 0.18 b	8.21 ± 0.36 b

Data are expressed as means ± SE.
Different symbols (a, b and c) show statistical significance between groups in each row.
AST: Aspartate Amino-Trasferase; ALT: Alanine Amino-Transferase; Cu: Copper

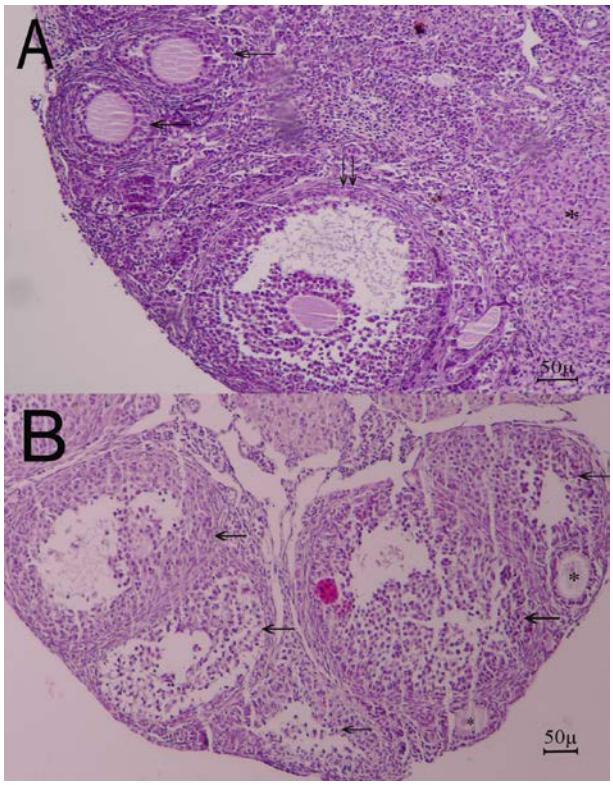

Fig 2. **(A).** Control group. Many different stages of normal developing follicles, growing follicles (arrows), antral follicle (double arrow), and corpus luteum (star). **(B).** Group 200 after 35th day. Many atretic follicles (arrows) and normal growing follicles (stars). H&E. (100×)

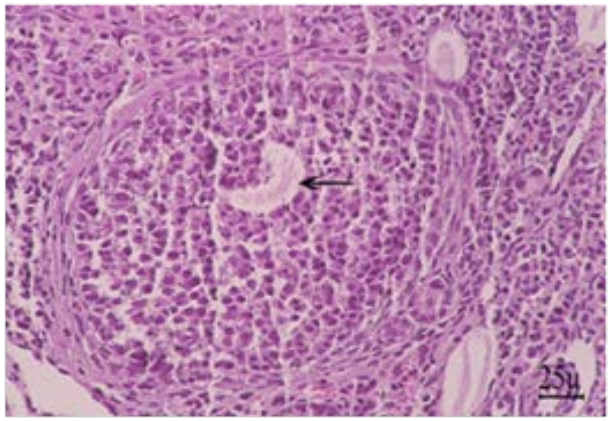

Fig 3. Ovary. Group 200 after 35th day. Atretic follicle with degenerated oocyte and desquamated pyknotic granulosa cells. H&E. (400×)

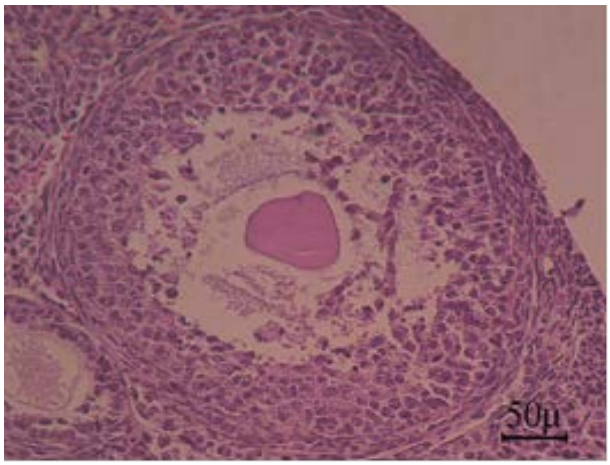

Fig 4. Ovary. Group 200 after 35th day. Degenerating antral follicle with desquamation of pyknotic granulosa cells and degenerated oocyte. H&E. (400×)

Ovarian lesions were observed in group 100 after 35th day and in group 200 after 21st day of experimental period. The histopathologic examination revealed widespread ovarian follicle atresia in group 200 after 35th day of experimental period (Fig. 2).

Table 2. Levels of serum AST, ALT and copper and liver copper concentration following administration of copper sulfate at a dose of 200 mg kg^{-1} per day for 35 days.

Parameters	Control group	Group 200		
		7th day	21st day	35th day
AST (IU L^{-1})	75.40 ± 1.25 a	77.20 ± 0.72 a	90.80 ± 0.80 b	214.00 ± 2.36 c
ALT (IU L^{-1})	159.00 ± 1.61 a	176.00 ± 1.58 a	217.00 ± 1.39 b	560.00 ± 9.30 c
Serum Cu (µmol L^{-1})	14.3 ± 0.18 a	14.10 ± 0.12 a	68.80 ± 0.98 b	82.40 ± 1.23 c
Liver Cu (mmol kg^{-1})	4.65 ± 0.06 a	4.68 ± 0.09 a	13.70 ± 0.14 b	15.80 ± 0.17 c

Data are expressed as means ± SE.
Different symbols (a, b and c) show statistical significance between groups in each row.
AST: Aspartate Amino-Trasferase; ALT: Alanine Amino-Transferase; Cu: Copper

Atretic follicles had numerous cell debris and inflammatory cells in the antral cavity (Fig. 3). In degenerative antral follicles granulosa cells degenerated and desquamated into antrum. In some degenerative antral follicles infiltration of inflammatory cells into granulosa cell layer and antrum were observed (Fig. 4).

Discussion

Copper is an essential trace element as an integral component of many enzymes and proteins, and is needed in a wide range of metabolic processes. The biological functions of copper include electron-transfer catalysis by means of its two accessible oxidation states.[5]

The toxicity of copper may come about by abnormally increasing the concentration of this ion, since toxicity depends on the concentration of the metal ion.[6] Chronic copper toxicity primarily affects the liver because this is the first site of copper deposition after it enters the blood. Copper toxicity is typically manifested by the development of liver cirrhosis with episodes of hemolysis and damage to other organs.[7,8]

In a clinical plasma examination, AST and ALT activities in plasma represent biomarkers for liver functions.[9] Alterations of AST and ALT activities are liver-specific and have been used as a tool to study varying cell viability and changes in cell membrane permeability.[10] The present study demonstrates that in the copper-loaded mice there was a significantly increased activity of plasma ALT and AST ($P < 0.05$ and $P < 0.05$, respectively). Based on these observations, alterations in plasma AST and ALT activities (Table 1) reflect adverse effects of copper intake on the hepatic function. Results of previous studies showed that AST and ALT activities in the plasma of rats were significantly elevated by copper, indicating copper-related injury to the liver.[11,12]

One of the best-known consequences of excess copper is peroxidative damage to membrane lipids. Lipid peroxidation occurs by the reaction of lipid radicals and oxygen to form peroxy radicals.[13,14] Oxidative stress occurs when formation of reactive oxygen species (ROS) exceeds the ability of the cells to defend themselves from increased ROS. When oxidized molecules overwhelm the cell system, a cascade of detrimental effects ensues affecting the whole organ at biochemical and molecular levels (lipids, proteins, DNA, and/or RNA).[15] Often, there is increased cellular ROS when cells do not have enough defensive levels/activity of antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPX), and catalase (CAT). Previous work has demonstrated that enzymes that protect against oxidative stress are important for ovarian function.[16,17] SOD is one of the most important enzymes that function as a cellular antioxidant. SOD rapidly converts O_2 into H_2O_2, which is removed by catalase.[18] Zhang *et al.*, studied the effect of copper overload on hepatic lipid peroxidation and antioxidation defense capacity by overloading rats with copper sulfate orally (500 mg kg-1 body weight) 5 days a week. They found that excessive copper accumulation in the liver depressed SOD activity in serum and liver homogenates.[19]

Although copper toxicosis has been well known, the relationship between copper accumulation and oxidative stress in ovary has rarely been examined. Sayre *et al.*, suggested that excessive tissue accumulation of redox-active transition metals (e.g., Cu, Fe) can be cytotoxic, in particular because perturbation in metal homeostasis results in an array of cellular disturbances characterized by oxidative stress and increased free radical production.[20] However, the study of excess copper causing damage to some organs by decreasing antioxidants and increasing lipid peroxidation products may have important implications for the understanding of toxic processes in reproductive diseases. Serving as a cofactor of many enzymes, copper is essential to the life of cells; however, if copper ions are not properly transported, stored, and utilized, redox reactivity leads to the risk of damage to cells and tissues.[21]

To the best of our knowledge, this is the first study demonstrating that copper poisoning induces oxidative damage in the mouse ovary. Antral follicles are essential for female reproduction because they contain the oocytes necessary for fertilization, and they synthesize and secrete the hormones required for menstrual/ estrous cyclicity, maintenance of the reproductive tract, and fertility.[22] To date little is known about the mechanisms by which copper poisoning induces atresia of follicles, and may be ions from metals such as copper exhibit high affinity for thiol groups and may therefore severely disturb many cell metabolic functions.[23] Consequently, oxidative stress that is the state of redox disequilibrium in which ROS production overwhelms the antioxidant defense capacity of the cell, may lead to adverse biological consequences such as damage to lipids, DNA or proteins resulting in excess cell proliferation and apoptosis.[24] Our histopathological observations revealed that there is a time similarity between liver toxicity and ovarian damages. In our study ovarian damage with many atretic follicles were observed following hepatic toxicity. Possibly follicles are so sensitive to excess copper. In a previous report the copper content was higher in the follicular fluid and granulosa cells from all categories of atretic follicles.[25] Also, elevated levels of copper are associated with increased epinephrine and dopamine synthesis in brain and release of neurotransmitters is influenced by this ion.[26] In a report, the higher levels of copper altered neurotransmitters that had cumulatively led to induction of atresia.[25]

Another possible role for the mechanism of copper poisoning on follicular atresia could be by the act of copper

at the level of hypothalamus through the modulation of neural activity, modification of GnRH granules stability and modulation of neurohormone release. Copper ions have been shown to stimulate both basal and GnRH-stimulated LH release from pituitary cells of immature female rats *in vitro*. Copper is also an extremely potent releaser of GnRH from isolated hypothalamic granules, which supports the hypothesis that copper influences GnRH neurons and copper action only occurs in GnRH granules.[27] On the other hand the excess of the copper could also cause a dysfunction of estrogen receptors resulting in reproductive disorders.[28] We could not find any information about the effects of excess copper on GnRH receptors. An adverse effect of chronic copper poisoning on GnRH receptors could be taken into consideration that needs more research.

Conclusion

The histopathologic examination revealed widespread ovarian follicle atresia. Atretic follicles had numerous cell debris and inflammatory cells in the antral cavity. The histopathlogic data indicated the possibility of adverse effects of copper poisoning on the mice ovarian tissue. Copper might be mediator of the effect of oxidative damage and play an essential role in reproductive system.

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