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# The effects of copper toxicity on histopathological and morphometrical changes of the rat testes

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ABSTRACT

**Objective:** Exposure to environmental toxicants such as copper has been suggested to have adverse effects on male reproduction. Therefore, our aim in the present study was to investigate morphometrical changes of rat testes following long term consumption. **Methods:** Animals were divided into three experimental groups. Two different doses of copper sulfate were applied once a day for 8 weeks by gavage. The first treatment group received copper sulfate at a dose of 100 mg/kg (Cu100 group) and the second treatment group was given copper sulfate at a dose of 200 mg/kg (Cu200 group). Control animals received normal saline using the same method. Testes from five cases of 15 animals of each group were removed for histopathological examinations on days 14, 28 and 56. Morphometrically, seminiferous tubules diameter, spermatogonial cells nuclei diameter, sertoli cells nuclei diameter and epithelial height were measured in the experimental groups. Meiotic index and the percentage of spermatogenesis were also calculated. **Results:** The mean values of about mentioned morphometrical parameters in copper treated groups showed significant decrease on 14th day compared to the control group. Copper administration caused a significant damage to morphometrical parameters on 28th day compared to the day 14. Also, in some parameters further decreases were observed specially in the Cu200 group on 56th day such as the diameter of seminiferous tubules, spermatogonial and sertoli cells nuclei and epithelial height of germinal layer ($P$<0.05). **Conclusions:** The results show that exposure to copper has the deleterious effects on morphometrical structure of testes which are appeared as early as two weeks.

## 1. Introduction

Copper is an important biological trace element which is necessary for different metabolic functions and enzyme activities such as catalase, peroxidase, and cytochrome oxidase, and is essential for the utilization of iron[1,2]. Nevertheless, its over-exposure might produce wide adverse effects in different physiological systems. Usually, occupational exposure to copper may lead to copper toxicosis in the industrial workers[3]. In animals, long-term intake of copper compounds of different origin is the most common form of copper poisoning. It means that the animals are reared closed to industrial plants, and ingest copper from industrial deposits through feed or from air throughout their entire life[4]. Copper regulation is controlled mainly by the liver, where it can be mobilized into the circulation or excreted via the bile[5]. In chronic copper poisoning, copper is gradually deposited in the liver without producing any significant sign. When the hepatic copper storage capacity is exceeded, it may result in hepatocellular necrosis and consequently the liberation of copper from the liver into the blood stream produces hemolysis, jaundice, and renal insufficiency[6]. Thus, copper is a strong oxidant, in which it could bind to cell molecules during the high load[5]. Consequently, it may generate highly reactive hydroxyl radicals and then affect some cellular functions. Hence, dietary copper overload in rats has produced the lipid peroxidation of mitochondrial membranes[7].

Study on workers exposed to electric welding revealed an increase in semen concentration of copper along with lowering in sperm count, sperm viability and semen volume[8]. In adult male rats, long term ingestion of copper adversely affects fertility and testicular weight[9] and recently, deleterious effects of copper poisoning on sperm quality of rats has been investigated[10]. Pathological

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features of copper toxicosis specially in organs such as liver, kidney, spleen, lung and intestine have been well demonstrated in animals[4,11-13], but according to the authors' knowledge, there is a lack of information about the copper toxicity on histopathological and morphometrical changes of adult male rat testes. So, the present study was undertaken to thoroughly investigate morphometric parameters of rat testicular tissue following long term copper consumption.

## 2. Materials and methods

### 2.1. Animals

Forty-five Wistar albino male adult rats (200-240 g) were purchased from Razi Research Institute of Kerman, Iran and kept in the Center of Laboratory Animal Care at the Veterinary Faculty of Shahid Bahonar University of Kerman, Iran for one week before treatment. The rats were housed in groups of five per cage and maintained under standard laboratory conditions (12 h light: 12 h dark and 22±2 °C) during the experimental period. During the study, the animals received water and pellet food (Javaneh Khorasan Co., Iran) ad libitum. All investigations were conducted in accordance with the Guiding Principles for the Care and use of Research Animals and were approved by the Animal Ethics Committee at the Veterinary Faculty of Shahid Bahonar University of Kerman, Iran.

### 2.2. Experimental design

Animals were randomly allocated to either control (Con, $n$=15) or two treatment groups each containing fifteen animals. To monitor the short and long-term effects of copper on testicular structure, two different doses of copper sulfate were applied once a day for 56 consecutive days by gavage. The first treatment group received copper sulfate at a dose of 100 mg/kg in 0.2 cc (Cu100 group, $n$=15) and the second treatment group was given copper sulfate at a dose of 200 mg/kg in 0.2 cc (Cu200 group, $n$=15). Control animals received normal saline using the same volume and similar method. The dose of copper sulfate used in our experiment was according to the previous study for producing of copper poisoning in rats[10]. Animals from each experimental group were sacrificed upon diethyl ether anesthesia (May & Baker Ltd., Dagenham, England) by cervical dislocation on days 14, 28 and 56 after the beginning of copper sulfate consumption, respectively and left testes were removed for histopathological examinations.

### 2.3. Histhopatological and morphometrical examinations

All specimens were fixed in Bouin's solution, embedded in paraffin wax, sectioned with 5 μm thicknesses, stained with haematoxylin and eosin (H&E) and examined blindly by an expert pathologist under a light microscope. Morphometrically, the mean seminiferous tubule diameter and epithelial height were measured in each testis. The ten smallest, roundest tubules were identified and measured with an ocular micrometer under light microscopy. Mean diameter, in microns, was then determined for each group[14]. The epithelium height was obtained with the same tubules used to determined tubular diameters. The average diameter of the spermatogonia and sertoli cells nuclei were measured from 30 cells for each testis[15]. The other parameter was the percentage of spermatogenesis. For this purpose, two hundred seminiferous tubules were examined under light microscopy. The presence of spermatozoa within the seminiferous tubule was considered as the evidence of spermatogenesis. Lack of spermatozoa even in the presence of orderly progression of primary and secondary spermatocytes was not considered as the evidence of spermatogenesis for the purpose of this experimental study[14].

### 2.4. Statistical analysis

Data were subjected to analysis by SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). All data were tested for homogeneity of variances by *Levene* static test. When the variances were homogenous, the different morphometrical data between the control and copper-treated rats on days 14, 28 and 56 were separately analyzed by One-way ANOVA[16]. Results were expressed as mean±SEM and values were considered to be statistically significant at $P<0.05$.

## 3. Results

### 3.1. Histopathological observation

Figures 1, 2 and 3 illustrate sections from testes of animals in the control, Cu100 group on 28th day and Cu200 group on 56th day, respectively. The seminiferous tubules of the control rats showed normal morphology with the presence of spermatozoa in their lumens (Figure 1). The testes of copper treated groups were accompanied by various degrees of degenerative changes depending on the doses and duration of copper administration. These degenerative changes included destruction of seminiferous epithelium, significant depletion of the germinal layers with the presence of vacuoles in the seminiferous epithelium. On 28th day, the most of the seminiferous tubules were degenerated and a few of them seemed to be relatively normal (Figure 2). On 56th day, nearly all the seminiferous tubules were completely degenerated as only a single layer of sertoli cells and spermatogonia was present (Figure 3).

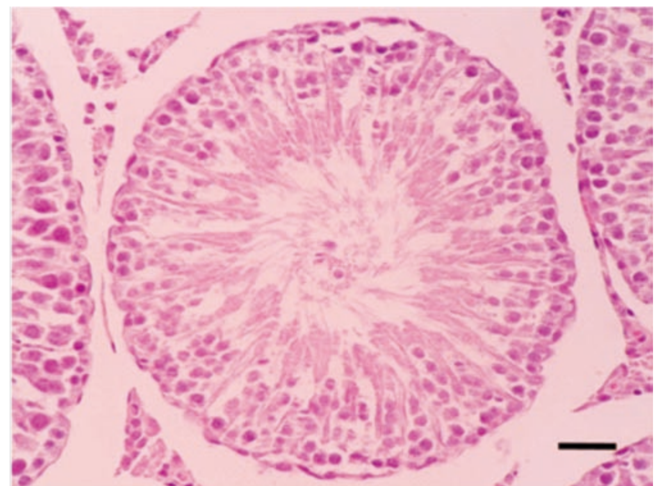

**Figure 1.** Control group. Photomicrograph showing normal seminiferous tubules morphology. H&E staining. Bar=30 μm.

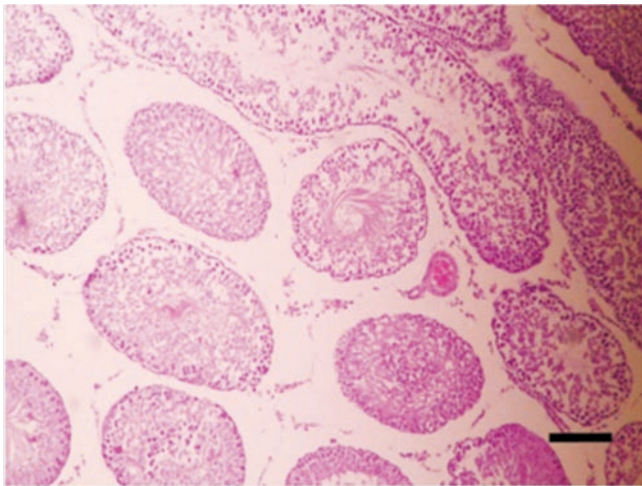

**Figure 2.** Copper treated group with a dose of 100 mg/kg (Cu100 group) on 28th day. Photomicrograph showing degenerated changes and a few relatively normal tubules. H&E staining. Bar=100 μm.

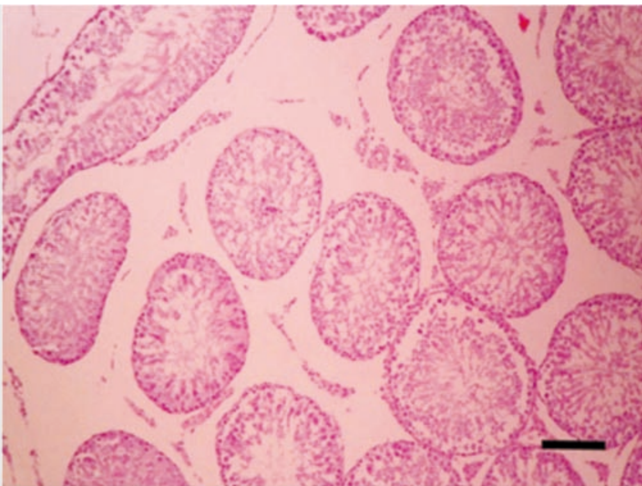

**Figure 3.** Copper treated group with a dose of 200 mg/kg (Cu200 group) on 56th day. Photomicrograph showing severe degeneration of seminiferous tubules along with depletion of germinal layer. H&E staining. Bar=100 μm.

### *3.2. Morphometrical evaluation*

#### *3.2.1. Spermatogenesis and meiotic index*

The mean percentage of spermatogenesis and meiotic index of the control and copper treated rats have been shown in tables 1 and 2. The mean percentages of spermatogenesis following treatment with 100 and 200 mg/kg of copper sulfate were 59.3% and 53.3% and the mean of meiotic index were 2.39 and 2.19 respectively which were significantly lower than that of the control group (70.5% and 2.75, respectively) in the first 14 days. The percentage of spermatogenesis and also meiotic index in Cu100 group on 28th day were significantly decreased ($P$<0.05) in comparison to the control group and there were no significant difference between the Cu100 and Cu200 groups. Further decreases were observed on 56th day for both spermatogenesis and meiotic index parameters which were significant ($P$<0.05) in Cu100 group (42.7±1.39 vs. 51.00±1.64 and 1.10±0.14 vs. 1.81±0.17, respectively) and were not significant (P>0.05) in the Cu200 group (42.5±1.39 vs. 48.00 ±2.12 and 1.02±0.12 vs. 1.19±0.16, respectively).

**Table 1**

Mean±SEM percentage of spermatogenesis in testes of rats at 14, 28 and 56 days after copper consumption.

| Experimental groups | Days after copper consumption | | |
|---|---|---|---|
| | 14th day | 28th day | 56th day |
| Con | $70.5±1.60^{*a}$ | $67.30±2.02^{*a}$ | $69.6±1.45^{*a}$ |
| Cu100 | $59.3±2.22^{*b}$ | $51.00±1.64^{\phi b}$ | $42.7±1.39^{\#b}$ |
| Cu200 | $53.3±3.67^{*b}$ | $48.00±2.12^{*\phi b}$ | $42.5±1.39^{\phi b}$ |

Cu, Copper sulfate; Cu100, 100 mg/kg copper sulfate administration; Cu200, 200 mg/kg copper sulfate administration.
$^{a, b, c}$ At each column, different superscript alphabets show significant difference ($P$<0.05). $^{*, \phi, \#}$ At each row, different superscript signs show significant difference ($P$<0.05).

**Table 2**

Mean±SEM of meiotic index in testes of rats at 14, 28 and 56 days after copper consumption.

| Experimental groups | Days after copper consumption | | |
|---|---|---|---|
| | 14th days | 28th days | 56th days |
| Con | $2.75±0.16^{*a}$ | $2.96±0.12^{*a}$ | $2.77±0.13^{*a}$ |
| CU100 | $2.39±0.08^{*b}$ | $1.81±0.17^{\phi b}$ | $1.10±0.14^{\#b}$ |
| CU200 | $2.19±0.08^{*b}$ | $1.19±0.16^{\phi c}$ | $1.02±0.12^{\phi b}$ |

Cu, Copper sulfate; Cu100, 100 mg/kg copper sulfate administration; Cu200, 200 mg/kg copper sulfate administration.
$^{a, b, c}$ At each column, different superscript alphabets show significant difference ($P$<0.05). $^{*, \phi, \#}$ At each row, different superscript signs show significant difference ($P$<0.05).

#### *3.2.2. Seminiferous tubules diameter and epithelial height*

Table 3 shows the effects of copper sulfate administration on the mean diameter of seminiferous tubules in the left testes of rats at 14, 28 and 56 days following copper consumption. Administration of copper with a dose of 100 mg/kg (Cu100 group) caused a significant reduction in the mean diameter of seminiferous tubules on 28th day compared to the 14th day and the control group (242.00±4.87 vs. 329.00±5.69 and 330.00±11.98, respectively) and it did not have any significant difference with the 56th day. The mean diameter of seminiferous tubules in the Cu200 group showed significant reduction from 14th day and severe damage was observed on 56th day in comparison to the control group (197.50±3.57 vs. 327.69±13.01, $P$<0.05).

**Table 3**

Mean±SEM diameter (μm) of seminiferous tubules in testes of rats at 14, 28 and 56 days after copper consumption.

| Experimental groups | Days after copper consumption | | |
|---|---|---|---|
| | 14th day | 28th day | 56th day |
| Con | $330.00±11.98^{*a}$ | $320.76±7.71^{*a}$ | $327.69±13.01^{*a}$ |
| Cu100 | $329.00±5.69^{*a}$ | $217.00±8.58^{\phi b}$ | $204.50±4.15^{\phi b}$ |
| Cu200 | $242.00±4.87^{*b}$ | $214.00±4.12^{\phi b}$ | $197.50±3.57^{\#b}$ |

Cu, Copper sulfate; Cu100, 100 mg/kg copper sulfate administration; Cu200, 200 mg/kg copper sulfate administration.
$^{a, b, c}$ At each column, different superscript alphabets show significant difference ($P$<0.05). $^{*, \phi, \#}$ At each row, different superscript signs show significant difference ($P$<0.05).

The data following copper sulfate administration (Table 4) showed that the mean of epithelial height in groups 100 and 200 mg/kg on 14th day were significantly decreased

in comparison to the control group (77.42±1.33 and 72.99±1.35 vs. 98.80±2.67, respectively). Decrease in the epithelial heights was continued up to the day 28 for both the Cu100 and Cu200 groups ($P$<0.05) but only administration of 200 mg/kg copper (Cu200 group) caused a further reduction on the 56th day compared to the 28th day (62.41±1.32 vs. 68.47±1.42, $P$<0.05).

**Table 4**

Mean±SEM epithelial height (μm) of seminiferous tubules in testes of rats at 14, 28 and 56 days after copper consumption.

| Experimental groups | Days after copper consumption | | |
|---|---|---|---|
| | 14th day | 28th day | 56th day |
| Con | 98.80±2.67$^{*a}$ | 103.80±4.20$^{*a}$ | 101.80±2.58$^{*a}$ |
| Cu100 | 77.42±1.33$^{*b}$ | 70.78±1.31$^{\phi b}$ | 70.01±1.34$^{\phi b}$ |
| Cu200 | 72.99±1.35$^{*c}$ | 68.47±1.42$^{\phi b}$ | 62.41±1.32$^{\#c}$ |

Cu, Copper sulfate; Cu100, 100 mg/kg copper sulfate administration; Cu200, 200 mg/kg copper sulfate administration.
$^{a, b, c}$ At each column, different superscript alphabets show significant difference ($P$<0.05). $^{*, \phi, \#}$ At each row, different superscript signs show significant difference ($P$<0.05).

### *3.2.3. Spermatogonia and sertoli cells nuclei diameter*

The mean diameter of spermatogonia and sertoli cells nuclei of the control and copper treated rats on days 14, 28 and 56 after the beginning of copper consumption have been shown in tables 5 and 6. The mean diameter of spermatogonia and sertoli cells nuclei on 14th day were significantly decreased in the Cu100 and Cu200 groups in comparison to the control group ($P$<0.05) and further significant decreases were observed for both copper treated groups on day 28 compared to the 14th day ($P$<0.05). Reduction in the mean diameter of spermatogonia and sertoli cells nuclei were continued up to the day 56 ($P$<0.05). Thus, we could not find any significant difference between the Cu100 and Cu200 groups on 56th day for the mean diameter of spermatogonia cells nuclei (5.00±0.09 vs. 4.71±0.11, respectively) and sertoli cells nuclei (3.75±0.20 vs. 3.89±0.21, respectively).

**Table 5**

Mean±SEM diameter (μm) of spermatogonia cells nuclei in testes of rats at 14, 28 and 56 days after copper consumption.

| Experimental groups | Days after copper consumption | | |
|---|---|---|---|
| | 14th day | 28th day | 56th day |
| Con | 7.70±0.37$^{*a}$ | 7.15±0.27$^{*a}$ | 7.29±0.31$^{*a}$ |
| Cu100 | 6.85±0.18$^{*b}$ | 5.95±0.14$^{\phi b}$ | 5.00±0.09$^{\#b}$ |
| Cu200 | 6.46±0.19$^{*b}$ | 5.25±0.10$^{\phi c}$ | 4.71±0.11$^{\#b}$ |

Cu, Copper sulfate; Cu100, 100 mg/kg copper sulfate administration; Cu200, 200 mg/kg copper sulfate administration.
$^{a, b, c}$ At each column, different superscript alphabets show significant difference ($P$<0.05). $^{*, \phi, \#}$ At each row, different superscript signs show significant difference ($P$<0.05).

**Table 6**

Mean±SEM diameter (μm) of sertoli cells nuclei in testes of rats at 14, 28 and 56 days after copper consumption.

| Experimental groups | Days after copper consumption | | |
|---|---|---|---|
| | 14th day | 28th day | 56th day |
| Con | 8.04±0.47$^{*a}$ | 7.95±0.53$^{*a}$ | 8.12±0.30$^{*a}$ |
| Cu100 | 6.78±0.23$^{*b}$ | 4.68±0.20$^{\phi b}$ | 3.75±0.20$^{\#b}$ |
| Cu200 | 5.93±0.30$^{*c}$ | 4.26±0.25$^{\phi b}$ | 3.89±0.21$^{\#b}$ |

Cu, Copper sulfate; Cu100, 100 mg/kg copper sulfate administration; Cu200, 200 mg/kg copper sulfate administration.
$^{a, b, c}$ At each column, different superscript alphabets show significant difference ($P$<0.05). $^{*, \phi, \#}$ At each row, different superscript signs show significant difference ($P$<0.05).

## 4. Discussion

The results of the present study clearly demonstrated that copper intake even with low dose (100 mg/kg) is able to have toxic effects from 14$^{th}$ day on testes morphometrically. In our previous report, adverse effects of copper administration on mice ovaries were observed after 21th day at a dose of 200 mg/kg and after 35th day at a dose of 100 mg/kg[17]. This observation is in contrast to our present study and so it can show sensitivity of testicular tissue to environmental toxicants. Interestingly, in another report by Sakhaee *et al*., 2012[10], significant increase in rats serum copper level was observed at a dose of 200 mg/kg on 42th day. It means that testicular damage has been happened before rising in serum copper level. The possible explanation for these changes by copper in such a short period of time can be due to participation of copper ions in the formation of reactive oxygen species (ROS)[5]. Copper is a strong oxidant in which it could bind to cell molecules during the high load[5]. Cupric ions coming from the corrosion of metallic copper can be reduced to cuprous ions in the presence of biological reductants[18]. Cuprous ions is able to catalyze the formation of ROS through the decomposition of hydrogen peroxide ($H_2O_2$) via Fenton/Haber-Weiss reaction[19,20]. In addition, ions from metals such as copper exhibit high affinity for thiol groups and may therefore severely disturb many cell metabolic function[21]. 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, apoptosis, or mutagenesis[22]. Mammalian spermatozoa membranes are very sensitive to the damage mediated by lipid peroxidation, because they are rich in polyunsaturated fatty acids in plasma membrane[23]. The fatty acids are an essential requirement for the male germ cell to maintain sperm functions[24]. Therefore, germ cells are as vulnerable as other cells to the potential detrimental effects of ROS[25].

In addition, in the present study we showed that administration of copper damaged sertoli cells as well as spermatogonia and these could be one of the explanation for impaired spermatogenesis and meiotic index. So, the other explanation for these wide cellular damage could be activation of apoptosis process and oxidative damage to DNA of cells[26]. Apoptosis is associated with specific morphological changes which are characterized by chromatin condensation, nuclear DNA fragmentation, cell shrinkage and membrane-enclosed cell fragment (apoptic body) formation[27]. Several researchers have reported copper cytotoxicity in a variety of in vitro and in vivo systems. Wataha[28]observed that the reduction of cell proliferation and increased frequency of nonviable cells are dependent on copper concentration. It has also been shown that copper compounds delay cell-cycle progression and increase cell death in different cell cultures[29-31]. Previous investigations provide evidence that copper ions are capable of interacting directly with nuclear proteins and DNA causing site-specific damage[32]. Copper easily reacts with both individual amino acids and proteins containing histidine and cysteine. Complexes of copper ion with amino acids or peptides are able to bind to DNA forming copper-amino acid-DNA complexes which in consequence leads to constant change of the structure genes. Modulation of transcription is exerted by copper's influence on the properties of the transcription factors[33]. Copper binds to DNA with higher affinity than

other cations and thus promotes DNA oxidation[34], catalyses efficient formation of 8-aminodeoxyguanosine as well as 8-oxidG[35], and causes apoptosis in cultured cells[36,37].

In conclusion, this work provided evidence for deleterious effect of copper on testes as early as two weeks. Copper poisoning affected different cell type such as spermatogonia and sertoli cells which lead to decrease in the percentage of spermatogenesis.

## Conflict of interest statement

The authors declare that there are no conflicts of interest.

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