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Original Article

Histopathological evaluation of supportive effect of vitamin C on testes following long-term administration of copper sulfate in mice

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Abstract
This study was set to investigate whether the adverse effects of long-term copper (Cu) consumption on testicular tissue could be prevented by vitamin C administration. Thirty-six sexually mature male NMRI mice were randomly divided into three groups of 12 animals. The first group (group Cu) which is treated by gavage with copper sulfate at a dose of 200 mg/kg/day (0.2 ml) and 0.1 ml of distilled water via intraperitoneal (i.p.) injection daily for 6 weeks, the treatment group (group Cu + Vit.C) which is treated by gavage with copper sulfate at a dose of 200 mg/kg/day (0.2 ml) and received 10 mg/kg/day (0.1 ml) vitamin C by i.p. injection, and the control group which received the same volume of distilled water by gavage and i.p. injection during the experimental period. Shrinkage and collapse of tubules, decreased number of germinal cells and decrease in epithelial height were histopathologial lesions of testes of Cu group, while, treatment group showed normal morphology with presence of spermatozoa in their lumen. In conclusion, vitamin C can be one of the suitable choices for preventive therapeutic effects and/or improvement in testicular tissue after Cu toxicosis.

INTRODUCTION

Copper is an essential trace element that is widely distributed in animal tissues [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, the 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].

Soluble copper salts in high concentrations are protein coagulants. The ingestion of large quantities is associated with intense irritation of the alimentary mucosa and profound shock. Severe intravascular hemolysis occurs if the animal survives long enough. When excessive amounts of copper are injected the response is rapid and animals begin to die the next day and with a peak of mortality about the third day after dosing. Early deaths appear to be due to severe hepatic insufficiency and later deaths due to renal failure. The frequent ingestion of small amounts produces no ill-effects while copper accumulates in the liver. When maximum hepatic levels are reached after periods of exposure often as long as 6 months, the copper is released into the bloodstream and the animal dies of acute intravascular hemolysis. One of the dangers of cumulative copper poisoning is that the animal shows normal health until the hemolytic crisis, when it becomes acutely ill and dies very quickly. Death is ascribed to acute anemia and hemoglobinuric nephrosis. Two other abnormalities have been observed during and after the hemolytic crisis. One is the

occurrence of methemoglobinemia; the other is the presence of degenerative lesions in the white matter of the brain. There are a number of explanations for the development of hemolysis. One is that the erythrocytes in affected sheep become immunogenic as a result of the copper accentuation. It is suggested that this immunogenicity leads to the development of an autoantibody and the final result of an autoimmune hemolytic anemia [5].

Alternatively, copper may act as an oxidant on the red cell membrane, leading to membrane damage and acute hemolysis as a result of oxidant injury. This is consistent with the formation of methemoglobin, a result of oxidant effects during the acute hemolytic crisis. The liberation of the hepatic copper is incompletely understood, but the favored hypothesis is that the accumulation of copper ions in the liver cells is associated with the accumulation of electron-dense Iysozymes in the hepatocytes and their eventual necrosis. Various stresses including a fall in plane of nutrition, traveling, and lactation, are considered to precipitate the liberation. Complex mechanisms relating to disorders of cell membranes, a marked change in hemoglobin composition, including the development of methemoglobinemia and an increase in the oxidative status, are described as occurring during the critical stages. During the prehemolytic stage of several weeks before the crisis there is hepatic necrosis and an elevation of levels of liver-specific enzymes. A much more serious necrosis of liver occurs at the time of the hemolytic crisis [5].

Exposure to environmental contaminants has been suggested to play a role in the pathophysiology of adverse reproductive health effects including decreased semen quality, subfertility, change in birth sex ratio, and an increase in the prevalence of developmental abnormalities of the male reproductive tract [6, 7]. The aim of the current study was to evaluate beneficial effects of vitamin C on testes following long-term administration of copper sulfate in mice.

MATERIALS AND METHODS

Thirty-six sexually mature male 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 Veterinary Medicine Faculty of Shahid Bahonar University of Kerman for 1 week before treatment. The mice weighed 25-30 g and were of the same age (1.5-2 months old). The experimental animals were randomly divided into four groups of 12 animals and were housed in standard polypropylene cages with wire mesh top, at 21°C in a 12/12 h dark/light cycle. During the study, the animals received water and pellet food (Javaneh Khorasan Co, Mashhad, Iran) *ad libitum*. All ethical considerations using animals were considered carefully and the experimental protocol was approved by the Ethics Committee of KUMS.

Experimental design

The study comprised three different groups of 12 mice as follows: the first group (group Cu) which is treated by gavage with copper sulfate at a dose of 200 mg/kg/day (0.2 ml) and 0.1 ml of distilled water daily via intraperitoneal (i.p.) injection for 6 weeks; the treatment group (group Cu + Vit.C) which is treated by gavage with copper sulfate at a dose of 200 mg/kg/day (0.2 ml) and received 10 mg/kg/day (0.1 ml) vitamin C by i.p. injection; and the control group which received the same volume of distilled water by gavage and i.p. injection during experimental period.

Histopathological assays

After the treatment procedure, total testes were excised from six cases of 12 animals of each group and preserved in 10% neutral buffered formalin solution for histological examination at fourth and sixth week. Formalin-fixed samples were processed by the standard paraffin wax technique, and sections of 5 μm thickness were cut and stained with hematoxylin and eosin.

Morphometrical assays

For morphometric assays, the seminiferous tubule diameter was measured in each testis. The ten smallest, roundest tubules were selected for each animal per group, and the diameter of tubules measured with an ocular micrometer under light microscopy. The number of round spermatids for each of the pachytene primary spermatocytes (meiotic index) was also calculated for determination of cell loss percentage during cell division [8]. Spermatogenesis was determined by the semiquantitative testicular biopsy score count in 100 cross-sections in each case at the same magnification and was summed up as mean Johnsen score (MJS) [9].

Statistical analysis

All data were expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed using one-way analysis of variance, followed by post hoc Tukey HSD test. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Tables 1, 2 and 3 show the number of round spermatids for each pachytene primary spermatocyte (meiotic index), the seminiferous tubule diameter and the semi-quantitative testicular biopsy score count (Johnsen score) in each case, respectively.

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Figs.1&2 illustrate sections from testes of animals in copper and treatment group, respectively. Histopathological lesions of testes of Cu group were included: shrinkage and collapse of tubules, decreased number of germinal cells and decrease in epithelial height (Fig.1). Also, the number of seminiferous tubules containing spermatozoa was decreased. The tubules lack spermatids and spermatozoa, and are lined by Sertoli cells, spermatogonia, and primary spermatocytes (Fig.1). The seminiferous tubules of treatment group show normal morphology with presence of spermatozoa in their lumen (Fig.2).

Table 1. Mean ± SEM of meiotic index in testes of mice at 28 and 42 days after treatment

Experimental groups	weeks after treatment	
	4th week	6th week
Control	2.29 ± 0.11^{a}	3.33 ± 0.06^{a}
Cu	1.65 ± 0.05^{ab}	1.56 ± 0.03^{a}
Cu + Vit.C	2.13 ± 0.09^{b}	2.57 ± 0.08^{a}

The same letters (a and b) for each column show statistical significance between the groups.

Table 2. Mean ± SEM diameter (μm) of seminiferous tubules in testes of mice at 28 and 42 days after treatment

Experimental groups	weeks after treatment	
	4th week	6th week
Control	216.67 ± 3.59	226.4 ± 4.72^{a}
Cu	208.3 ± 3.7	209.95 ± 3.07^{a}
Cu + Vit. C	210.8 ± 4.29	218.4 ± 2.62

The superscripted letter (a) indicates statistical significance among groups of this column.

Table 3. Mean Johnsen score in testes of mice at 28 and 42 days after treatment

Experimental groups	weeks after treatment					
	4th week			6th week		
	Mean ± SE	Range	Median	Mean ± SE	Range	Median
Control	8.68 ± 0.17^{a}	5 – 10	9	9.28 ± 0.09^{a}	7 – 10	9
Cu	7.48 ± 0.17^{ab}	5 – 9	7.5	7.56 ± 0.19^{a}	4 – 9	8
Cu + Vit.C	8.2 ± 0.21^{b}	5 – 10	8	8.36 ± 0.42^{a}	6 – 10	8

The same letters (a and b) for each column show statistical significance between the groups.

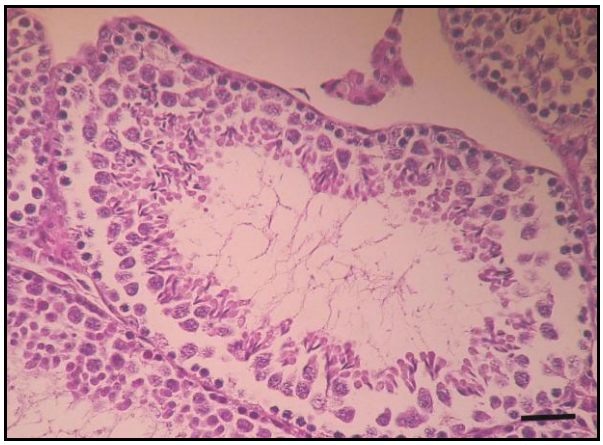

Figure 1. Cu administered group: shrinkage and collapse of tubules, decreased number of germinal cells and decrease in epithelial height. (H&E bar = 50 μm)

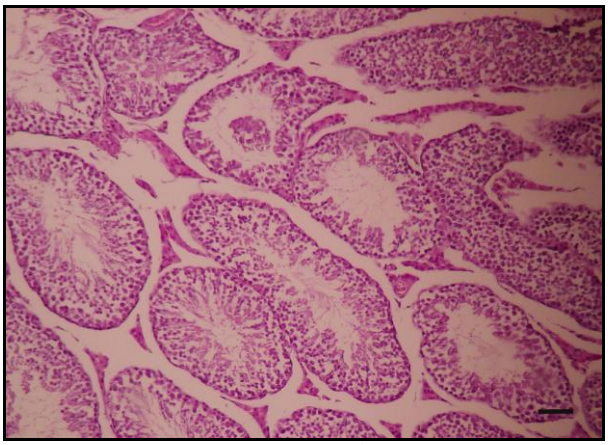

Figure 2. Cu + Vit.C group: the seminiferous tubules show normal morphology with presence of spermatozoa in lumen. (H&E; bar = 100 μm)

DISCUSSION

The role of copper in male reproductive capacity appears to be largely unknown, but this heavy metal appears to be involved in sperm motility and it may also act at the pituitary receptors, which control the release of LH [10].

It is known that copper is an essential trace element that plays an important role in several enzymes such as superoxide dismutase. Human spermatozoa are particularly susceptible to peroxidative damage because they contain high concentrations of polyunsaturated

fatty acids and also possess a significant ability to generate reactive oxygen species (ROS), mainly superoxide anion and hydrogen peroxide. Superoxide dismutase protects human spermatozoa from this peroxidative damage. Oxidative stress caused by accumulated ROS is closely involved in a variety of pathological processes. Germ cells are as vulnerable as other cells to the potential detrimental effects of ROS and may thus require antioxidant protection at sites of gamete production, maturation and storage, and embryo implantation [11]. It is reported that copper acts as a catalyst in the formation of ROS which can lead to oxidative stress and destructive lipid peroxidative damage [12]. Copper might be a mediator of the effect of oxidative damage and play an essential role in spermatogenesis and male infertility [13].

It has been shown that copper *in vitro* increased lipoprotein oxidation [14, 15]. Spermatozoa are highly susceptible to damage by excess concentrations of ROS due to the high content of polyunsaturated fatty acids within their plasma membrane and, although conventional basic semen characteristics other than motility are not obviously influenced by the oxidative state of semen [16], such damage may underlie several aspects of male infertility. Increased lipid peroxidation and altered membrane function can render sperm dysfunction through impaired metabolism, motility, acrosome reaction reactivity and fusogenic capacity as well as oxidative damage to sperm DNA [17].

Results of previous studies showed the beneficial effects of vitamin C on epididymal sperm quality following experimentally induced copper poisoning in mice [18]. This study demonstrates that the spermatogenic damage due to long-term administration of copper can be improved by vitamin C therapy. In our experiment, the spermatogenic process reduced after copper poisoning because of damage to the germ cells, the pachytene spermatocytes and early spermatids. As the length of time from initiation of stem cell division to formation of spermatozoa is around 35 days for mice [19], the chosen period of time (6 weeks) provided sufficient time to monitor the potential recovery of spermatogenesis in surviving spermatogonia. Previous investigations have proved that copper overload can cause cellular damage by different mechanisms.

The pathological manifestations observed during this study are indicative of copper toxicosis [20]; similar results have been described in naturally occurring cases in young animals [21]. The data obtained show that copper sulphate at a dose of 200 mg/kg/day after 6 weeks that caused frank testicular atrophy correspondingly reduced sperm density in epididymal lumen, lowered motility and induced structural abnormalities in spermatozoa. The deterioration of seminiferous germinal epithelium or a spermicidal effect of copper sulphate may be responsible for such effects.

Vitamin C is one of several antioxidant vitamins, which have been postulated to minimize testicular cytotoxic effects in animals treated with pesticides, chemical mutagens, xenobiotics, and metals [22, 23]. The exact mechanism of the protective action of vitamin C against ROS-induced toxicity is not in detail understood. However, it is believed that vitamin C, as an antioxidant, might prevent the production of mutagenic electrophilic metabolites [24] and stimulate 7-α-hydroxylation of lipids and cholesterol nuclei, thus enhancing their degradation to bile acids, which could be excreted from the body. Alternatively, vitamin C, as a part of a redox buffer system, can effectively scavenge harmful ROS [25]. Such antioxidant action of vitamin C could relieve the male germ cells from oxidative damage, thereby increasing sperm count and decreasing the percentage of abnormal sperm population.

Considering the nontoxic and safe nature of vitamin C, it can be one of the suitable choices for preventive therapeutic effects and/or improvement in testicular tissue after Cu toxicosis. More research is needed to reveal the exact mechanism of vitamin C on the hormonal regulation of the testes and epididymides.

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