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Temporal kinetics of organ damage in copper toxicity: A histopathological correlation in rat model

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ABSTRACT

Excess of copper is toxic to different organs. We aim to study the histopathological changes of liver, kidney, and brain following oral CuSO4 exposure for different duration and doses in rat model. Fifty-four males Wistar rats (205 ± 10 g) were included and divided into control (group-I) and experimental (group-II and III) arms. An oral dose of 100 and 200 mg/kgBWt/Day CuSO4 was given to group-II and III respectively and group-I received normal saline by gavage. Six rats from each group were sacrificed on days 30, 60 and 90 for biochemical and histopathological examinations. The histopathological changes were graded on 1–5 scores and correlated with respective laboratory parameters. The organ functions were worsened in experimental group with increasing dose and time. Histopathological study revealed edema, hemorrhage, necrosis and fibrosis/gliosis in experimental group. The worst histopathological severity score ranged from 4 to 5(median 5) in liver, 3–5(median 4) in kidney and 4–5(median 5) in brain. The edema and hemorrhage were more marked at 30 days and fibrosis/gliosis at 90 days. In conclusion, high-dose Cu toxicity results in structural damage to liver, kidney, and brain that correlates with organ dysfunction, Cu, GSH, TAC, and MDA concentrations. Liver damage is more severe and occurs earlier than other organs.

1. Introduction

Copper (Cu) is an essential element in human and animal and plays an important role as a catalytic co-factor for a range of metalloenzymes including Cu, Zn-superoxide dismutase, cytochrome-c oxidase, ceruloplasmin and dopamine beta-mono-oxygenase (Shim and Harris, 2003; Valko et al., 2005). A higher concentration of Cu however may be toxic to the cells due to its ability to directly react with molecular oxygen, leading to generation of hydroxyl radicals in a Fenton-like reaction (Halliwell, 2001; Youdim and Riederer, 1997). Cells usually have mechanisms to regulate the uptake, distribution, detoxification and excretion of Cu. Thus, an anomaly in homeostasis has been linked to diseases, which are either associated with Cu deficiency or Cu toxicity symptoms. For example, dysfunction of the P-type ATPase ATP7B, which intermediates the Cu export and transport of cellular Cu into the secretory pathway results in Wilson disease (WD) (Ala et al., 2007; Wu et al., 2015). In WD patients, hepatic manifestation is seen in the first decade of life and neurological involvement in the second decade. Increased serum Cu leads to the accumulation of Cu in the liver, brain, cornea, lens, kidney, and other tissues. Magnetic resonance imaging (MRI) of brain in WD revealed the involvement of basal ganglia, thalamus, sub thalamic, midbrain, pons, subcortical white matter and occasionally gray matter (Kalita et al., 2014, 2015; Page et al., 2004; Ranjan et al., 2015; Sinha et al., 2007). Copper-intoxicated rats demonstrated increased deposition of Cu in the choroid plexus, swelling, increased size and number of astrocytes as well as, degenerated neurons and dense eosinophilic cytoplasm in the brain (Pal et al., 2013). Histopathology in Cu toxicity reveals fatty change, acute liver necrosis, chronic active hepatitis and macro-nodular cirrhosis (Cisternas et al., 2005; Narasaki, 1980; Ozcelik et al., 2003; Pal et al., 2013; Toyokuni et al., 1989). In the kidney, there is renal glomerular and tubular dysfunction, manifested by proteinuria, reduced glomerular filtration, amino aciduria, and phosphaturia. These abnormalities are attributed to Cu

Abbreviations: Cu, Copper; ALT, Alanine aminotransferase; AST, Aspartate aminotransferase; BUN, Blood urea nitrogen; CNS, Central nervous system; kgBWt, Kg Body Weight; LEC, Long-Evans Cinnamon; WD, Wilson disease.

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deposition in the renal tubules (Fuentealba et al., 1989; Haywood, 1980; Narasaki, 1980). Most of the above-mentioned studies were in experimental animal model focused on a particular organ rather than studying the temporal kinetics of different organs. Moreover, histopathological changes had not been correlated with respective organ function tests, Cu concentration and oxidative stress markers. Understanding the kinetics of Cu toxicity may be helpful in understanding the temporal profile of organ dysfunction in WD.

Therefore, we report the vulnerability of different organs to CuSO4 toxicity in rat model, and evaluate the histopathological changes of liver, kidney and brain and correlate these changes with tissue Cu and oxidative stress markers.

2. Material and methods

2.1. Animals and treatment

Fifty-four male Wistar rats (205 ± 10 g) were housed in the laboratory animal division of Central Drug Research Institute (CDRI), Lucknow, India and acclimatized for 10 days before starting the experiment. All animals were housed in the standard cage (6 rats/cage), feeding with a standard laboratory diet and tap water ad libitum. The experimental animals were housed in the air-conditioned room at 21–23 °C and 60–65% of relative humidity and kept on a 12 h light and dark cycle. All protocols were approved by Animal Ethics Committee of Central Drug Research Institute, Lucknow, India (IACE/2012/29).

The rats were randomly divided into 3 groups, including 6 rats in each group and fed the same diet by oral gavage throughout the experimental period. The experimental group received 100 mg/kgBWt (group-II) and 200 mg/kgBWt (group-III) of $CuSO_4$ daily up to 90 days, whereas the control group (Group-I) received saline water (Kumar et al., 2015, 2016).

2.2. Blood and tissue collection

At the end of the experiment, the overnight fasted animals were sacrificed under isoflurane anesthesia. 5.00 ml blood was drawn by cardiac puncture before incision of the abdomen and stored in plain and heparin tubes. Serum and plasma were separated and frozen at −80 °C until the time of analysis. Liver, brain, and kidney tissues were cut in small pieces and immersed in neutral 10% buffered formalin for histopathology.

2.3. Measurement of Cu

Cu content in the liver, brain, and kidney tissue were measured using a flame type atomic absorption (GBC Avanta Sigma, GBC Scientific equipment PTY Ltd, Dandong, Victoria Australia). Briefly, 0.5 g of each organ was digested with a mixture of nitric acid (HNO_3) and perchloric acid ($HClO_4$) (6:1, v/v) and the digest was brought to constant volume with double distilled deionized water. Ceruloplasmin level was measured in tissue homogenate by its oxidase activity with o-dianisidine dihydrochloride following the method of Schosinsky et al. Samples were mixed with the optimal concentration of odianisidine dihydrochloride (7.88 mM) in 0.1 M acetate buffer (pH 5.0), and the absorption at 540 nm was measured (Schosinsky et al., 1974). The amount of Cu associated with ceruloplasmin is approximately 3.15 μg of Cu per mg of ceruloplasmin. Free Cu was estimated by subtracting 3.15 time of ceruloplasmin (mg/dl) from the total tissue Cu level (μg/dl).

2.4. Neurobehavioral study

Neurobehavioral study included grip strength, rotarod test, and Y-maze. These studies were done at baseline, 30, 60, and 90 days of experimentation one day before they were sacrificed.

2.4.1. Grip-strength test

A grip strength meter (TSE-Systems, Bad Homburg, Germany) was used to assess neuromuscular function by sensing the peak amount of force an animal applies in grasping a specially designed pull bar assembly. In practice, each rat was gently placed on the mesh and pulled by holding the tail in the opposite direction until it released its grip from the mesh. The peak force developed before the release of grip was recorded in Newton (N). We calculated the mean of five measurements, allowing 30 s of recovery time in between the test.

2.4.2. Y-maze test

Y-maze was used for assessing learning and memory. Y-maze consists of three arms with an angle of 120° between each arm. The maze was placed in a separate room with minimal light, and the floor of the maze was dusted with sawdust after each trial to eliminate olfactory stimuli. Two trials were done and the inter-trial interval was 1 h. The first trial (training) was for 10 min, and the rat was allowed to explore only two arms (starting arm and the other arm). For the second trial (retention), the rat was placed at the same starting arm and allowed to explore for 5 min with free access to all three arms. All trials were analyzed for the number of entries that the rat made into each arm. The results were expressed as the percentage of novel arm entries made during the 5-min retention trial.

2.4.3. Rotarod test

Rotarod was used for the assessment of motor coordination and balance using an accelerating rotarod apparatus (TSE-Systems, Bad Homburg, Germany). It was carried out by placing a rat on a rotating drum and measuring the length of time the animal was able to maintain its balance while walking on top of a rod. The rats were placed on the revolving rod accelerating at 5–50 rpm. Prior to testing, the rat was trained for at least three times and then allowed to rest for 15 min in between the trial. The apparatus was wiped with 70% ethanol and dried before each trial. The latency to fall from the rod was recorded, and any rat remaining on the rod for more than 300s was removed and returned to the cage.

2.5. Biochemical analysis

Hemoglobin, blood urea nitrogen (BUN) and serum bilirubin, transaminases and creatinine were measured using clinical chemistry analyzer (Randox Imbola, Ireland, United Kingdom).

2.6. Assay of glutathione (GSH), total antioxidant capacity (TAC) and malondialdehyde (MDA) level

2.6.1. Glutathione (GSH)

Tissue GSH level was measured according to the method described by Ellman. Tissue homogenate was added to 10% trichloroacetic acid (TCA), and allowed to stand at 4 °C for 2 h. This mixture was centrifuged at 3000 × g for 15 min and the supernatant was added to 2 ml of Tris buffer (0.4 mM, pH 8.9) containing EDTA (0.02 M) followed by the addition of 5,5′-Dithiobis-(2-Nitrobenzoic Acid) (0.01 M). Absorbance of yellow color product (5-thio-2-nitrobenzoic acid (TNB)) was read on a spectrophotometer at 412 nm (Ellman, 1959; Hasan and Haider, 1989).

2.6.2. Total antioxidant capacity (TAC)

Tissue TAC was measured by the method described by Koracevic. In this method tissue homogenate was added to a mixture of