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Relationship of antioxidant and oxidative stress markers in different organs following copper toxicity in a rat model

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ABSTRACT

Copper (Cu) at a higher level becomes toxic and it can catalyze the formation of highly reactive hydroxyl radical. We report the vulnerability of liver, kidney and brain to different dose of copper sulfate ($CuSO_4$) induced oxidative stress at different time duration. Fifty-four male Wistar rats (weight range = 205 ± 10 g) were equally divided into three groups. $CuSO_4$ was administered orally to the experimental groups (Group-II and III) up to 90 days in a dose of 100 and 200 mg/Kg body weight per day. Saline water was given to the control group (Group-I). At the end of 30, 60 and 90 days of administration, neurobehavioral studies were done and six rats from each group were sacrificed. Their liver, kidney and brain tissues were subjected for Cu, glutathione (GSH), malondialdehyde (MDA) and total antioxidant capacity (TAC) assay. Blood urea nitrogen (BUN), serum creatinine, bilirubin and transaminases were measured. GSH, TAC and MDA levels were correlated with the markers of respective organ dysfunction.

Administration of $CuSO_4$ resulted in increased free Cu and MDA level, and decrease GSH and TAC levels in group-II and III compared with group-I. In experimental groups, the reduction in TAC and GSH levels was maximum in liver tissue followed by brain and kidney; whereas increase in MDA level was highest in liver followed by brain and kidney at 30, 60 and 90 days. TAC and GSH levels in the liver inversely correlated with serum transaminases and bilirubin, and tissue free Cu, and positively correlated with MDA levels. Free Cu level in kidney tissue and BUN inversely correlated with TAC and GSH, and positively with MDA level. Grip-strength, rotarod and Y-maze findings were inversely correlated with brain free Cu and MDA levels and positively with GSH and TAC levels.

The oxidative stress was highest in liver followed by brain and kidney after oral $CuSO_4$ exposure in a rat model. These levels correlated with the respective organ dysfunction and tissue free Cu concentration.

1. Introduction

Copper (Cu) is an essential trace element absorbed from the diet through the small intestine. In the bound form, it reaches the liver where it can be stored within the hepatocyte, secreted into the plasma and excreted into the bile. In serum, around 65–90% of Cu firmly binds with ceruloplasmin, and the rest of Cu binds with albumin, transcuprein and amino acids (Linder and Hazegh-Azam, 1996; Linder et al., 1998; Valko et al., 2015). Cu is essential for mitochondrial respiratory chain, anti-oxidative defense and iron metabolism. It acts as a cofactor of redox-regulating enzymes, such as superoxide dismutase, ceruloplasmin, lysyl oxidase, tyrosinase, and dopamine β-hydroxylase. Cu also participates in nerve myelination and endorphin action (Linder and Hazegh-Azam, 1996; Shim and Harris, 2003; Valko et al., 2005; Turnlund, 1998; Gaggelli et al., 2006; Brewer, 2010). Excess of free Cu resulted in intracellular and extracellular Cu deposition in various organs. Free Cu is redox active and catalyzes the production of hydroxyl radicals in a Fenton-like reaction inducing oxidative stress and thereby cell injury (Linder and Hazegh-Azam, 1996; Valko et al., 2015; Boveris et al., 2012; Jomova and Valko, 2011; Jomova et al., 2010). Excess of Cu have a negative impact, because of its ability to generate free radical species that leads to oxidative stress. Wilson disease (WD) is an inherited metabolic disease due to ATP7B gene mutation in which there is defective Cu transport from the cells resulting in accumulation of Cu in liver, cornea, red blood cell and brain. In WD patients, urinary Cu is elevated above 100 μg/day (normal range 20–50 μg/day) and serum free Cu is increased over 25 μg/dl (normal range 8–12 μg/dl) and serum ceruloplasmin is decreased (<20 mg/dl). (Linder and Hazegh-Azam, 1996; Linder et al., 1998; Kalita et al., 2014; Kalita et al., 2015a). Central nervous system (CNS) is more vulnerable to oxidative stress because of low level of antioxidant (glutathione and catalase), and high flux of reactive oxygen species (ROS) produced during neurochemical reaction (dopamine synthesis) and high content of easily

Abbreviations: Cu, copper; GSH, glutathione; TAC, total antioxidant capacity; MDA, malondialdehyde; LPO, lipid peroxidation; 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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oxidizable substance (membrane polyunsaturated lipids) (Boveris et al., 2012; Jomova et al., 2010; Bruha et al., 2012; Cisternas et al., 2005; Nagasaka et al., 2009; Gaetke and Chow, 2003). The tripeptide glutathione (GSH) is a potent water-soluble antioxidant and is widely available in all tissues. GSH is the most important antioxidant and redox regulator in cells is essential in combating oxidation of cellular constituents. It also helps to keep protein in a reduced state and can suppress Cu toxicity by directly chelating the Cu (Mattie and Freedman, 2004). Unsaturated fatty acids are particularly susceptible to oxidative modification, and lipid peroxidation (LPO) is a sensitive marker of oxidative stress. LPO is a result of attacks by radicals on the double bond of unsaturated fatty acid, leading to generation of highly reactive lipid peroxyl radicals that initiate a chain reaction of further attacks on other unsaturated fatty acid. Low level of GSH, total antioxidant capacity (TAC) and high level of malondialdehyde (MDA) and other oxidative stress markers have been reported in brain and liver tissue in experimental model of 'Cu toxicity' e.g. Long-Evans Cinnamon (LEC) rat and toxic milk mice (Ozcelik et al., 2003; Ozcelik and Uzun, 2009; Pal et al., 2013a; Pal et al., 2013b; Joseph et al., 2009; Samuele et al., 1741; Leiva et al., 2009; Palm et al., 1990). The relationship of dose and duration of high sub-lethal dose of $CuSO_4$ exposure in the rat model revealed least Cu accumulation in the kidney and highest in the liver (Kumar et al., 2015). These changes may also reflect different levels of oxidative stress markers in different organ at different dose and time of Cu exposure. These may help in understanding the basis of cellular injury not only in dietary Cu toxicity, but also in inherited disease like Wilson disease (WD) (Kalita et al., 2014; Kalita et al., 2015b). In this experimental study using rat model, we report antioxidant (GSH, TAC) and oxidative stress (MDA) levels in liver, kidney and brain tissue and their respective organ dysfunction exposed to different dose of $CuSO_4$ for different duration of time.

2. Materials and methods

Six to eight months old, male Wistar rats weighing about 205 ± 10 g were allowed to acclimatize for 7 days prior to initiation of the study. The animals were housed in completely controlled environment (room temperature: 25 ± 2 °C and 12 h light and dark cycle) with free access to water and food. The feed was prepared locally and the Cu content was 5 mg per kg of diet (Council NR, 1995). The animal ethics committee of the Central Drug Research Institute, Lucknow, India approved the study protocol (IACE/2012/29).

2.1. Study design

Fifty-four male Wistar rats were divided into three groups. Each group consists of 18 rats. Group-I was designated as the control group and they received saline water orally by gavage. Group-II and III rats were designated as the experimental group and received $CuSO_4$ solution orally in a dose of 100 and 200 mg/kgBWt/day respectively by gavage. Rats were observed regularly for any abnormal clinical signs. Body weight and behavioral studies were done on day 0 and repeated on 30, 60, and 90 days of $CuSO_4$ exposure. Six rats from each group were sacrificed on days 30, 60 and 90. At the end of each exposure period, control and experimental rats were deeply anesthetized and exsanguinated by cardiac puncture. Blood samples from all the animals were drawn through cardiac puncture and were collected in sterile heparinized vials. Subsequently liver, kidney and brain were removed, transferred in liquid nitrogen to store at −80 °C until analyzed. Hemoglobin, blood urea nitrogen (BUN), serum bilirubin, transaminases and creatinine were measured. Ceruloplasmin 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). Total Cu concentration was measured in liver, kidney and brain tissue using atomic absorption spectroscopy (Kumar et al., 2015). Neurobehavioral studies such as grip strength, rotarod test and Y-maze were done at baseline and 30, 60 and 90 days of experimentation by using a standard method as described previously (Kumar et al., 2015).

2.2. Measurement of antioxidant and oxidative markers

GSH, TAC and MDA levels were measured by using spectrophotometer.

2.2.1. Glutathione (GSH). 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 x 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 was read on a spectrophotometer at 412 nm. A standard curve of glutathione was plotted to determine the amount of glutathione in the tissue homogenate sample (Hasan and Haider, 1989; Ellman, 1959).

2.2.2. Total antioxidant capacity (TAC). Tissue homogenate was added to a mixture of sodium phosphate buffer (100 mmol/L, pH 7.4), sodium benzoate(10 mmol/L), acetic acid then we add a freshly prepared Fe–EDTA and H_2O_2 (10 mmol/L) and incubate for 60 min at 37 °C. Then added acetic acid and TBA (Thiobarbituric acid) and incubate for 10 min at 100 °C (in a boiling water bath) then cool in an ice bath. TBARS (bright yellowish-brown in color) was produced by the reaction of hydroxyl radical with benzoate and was measured by spectrophotometer at 532 nm (Koracevic et al., 2001).

2.2.3. Malondialdehyde (MDA). Tissue homogenate in 0.1 M phosphate buffered saline (10% *w*/*v*) was incubated with 8.1% sodium dodecyl sulfate (SDS, *w*/*v*) for 10 min at room temperature followed by the addition of 20% acetic acid. The mixture was centrifuged and supernatant was aspirated out. Thiobarbituric acid (TBA, 0.8%, *w*/*v*) was added in the reaction mixture after vortexing the contents of the tube. The tubes were kept in a boiling water bath for 1 h. MDA level was determined with the absorption coefficient of MDA-TBA complex at 532 nm using spectrophotometer (Ohkawa et al., 1979).

2.3. Statistical analysis

The GSH, TAC and MDA levels in the liver, kidney and brain tissue were compared within the group at 30, 60 and 90 days by using one-way ANOVA with Bonferroni post-hoc multiple comparison test. These levels were also correlated with Cu concentration, hemoglobin, blood urea nitrogen, serum bilirubin, transaminase and creatinine as well as with findings of behavioral studies using Spearman or Karl-Pearson correlation tests. The variable with two tailed p value of less than 0.05 was considered significant. The statistical analysis was done by using SPSS-16 or Graphpad prism-5 software.

3. Results

The baseline body weights of the rats in different groups were not significantly different ($p = 1.00$). The group-II and group-III rats had significant reduction in body weight compared to group-I. Free Cu concentration was higher in experimental groups compared to the control group, and was maximum in the liver tissue followed by kidney and brain (Fig. 1). Value of Grip-strength, Rotarod test and Y-maze data at baseline were almost equal between all the three groups (Supplementary Table 1). These parameters significantly changes with time and