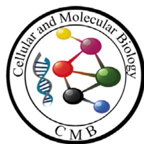

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Original Research

Chronic copper exposure elicit neurotoxic responses in rat brain: Assessment of 8-hydroxy-2-deoxyguanosine activity, oxidative stress and neurobehavioral parameters

Jayant Kumar, Kshirod Bihari Sathua, S. J. S. Flora*

National Institute of Pharmaceutical Education and Research (NIPER-R), New Transit Campus, Bijnor Road, Sarojini Nagar, Near CRPF Base Camp, Lucknow (UP)- 226002, India

Correspondence to: sjsflora@hotmail.com ; director@niperraebareli.edu.in

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Abstract: Copper (Cu), one of the essential transition metal acts as a prosthetic group for variety of proteins and metalloenzymes. However, it may be hazardous when administered in excess. Copper induced memory impairment and progression of neurodegenerative diseases have not yet been fully elucidated. The aim of the present study was to investigate the effect of exposure to copper sulphate (10mg/kg and 20mg/kg body weight, daily for 16 weeks) on brain copper concentration, few biochemical parameters indicative of oxidative stress and on different neurobehavioral functions in male Sprague Dawley rats. Copper-administered animals showed significant increase in brain copper concentration and a depleted Ceruloplasmin level. Different neurobehavioral studies revealed impaired memory and motor coordination in copper exposed rat. Spontaneous locomotors activity and depression symptoms were also noted in copper intoxicated rats. 8-hydroxy-2' -deoxyguanosine (8-OHdG) level, one of the predominant forms of free radical-induced oxidative lesions, and has been widely used as a biomarker for oxidative stress, increased in copper treated group. Copper induced oxidative stress in the brain was also evident from the increased lipid per oxidation (LPO) and nitrite level, depletion of reduced glutathione (GSH), and reduced activities of the antioxidant enzymes such as superoxide dismutase (SOD), and catalase. The present study thus suggests a significant correlation between copper induced oxidative stress and changes in neurobehavioral function in rats. The changes were more pronounced in animals exposed to a higher dose of copper (20mg/kg) than the lower dose.

Key words: Copper Toxicity; 8-OHdG; Oxidative Stress; Neurobehavioral; Rat.

Introduction

Copper (Cu) is an essential micronutrient that play a key role in the biochemistry of all aerobic organism. It serves as a prosthetic group for many enzymes such as superoxide dismutase (SOD1 and SOD3) (antioxidant defence), Cytochrome C Oxidase (electron transport in the mitochondrial respiratory chain), Tyrosinase (melanin synthesis), Dopamine beta-hydroxylase (catecholamine biosynthesis) (1-3). Copper absorption in mammals occurs from stomach and small intestine. After uptake it gets distributed within the different cells, detoxification and elimination in the body is maintained by the elegant system which make sure a regular and adequate supply of the micronutrient whereas concurrently avoiding excess levels (4). Copper is a prominent ecotoxicant and human being gets exposed to copper via consumption of water, food and also by inhalation of industrial dust (5-6). Recent reports suggest that agriculture workers handling of Cu-derived agrochemicals and also Cu-IUDs used by women lead to high plasma levels of copper (7). Excessive amounts of copper have been shown to abnormally accumulate in the targeted organ which is potentially hazardous to human health including imbalance in copper homeostasis.

The best depicted human copper related toxicosis disorder is Wilson disease (WD) which is an inherited disorder associated with the mutation in the Wilson disease protein (ATP7B) gene, leading to defective copper transportation and discharge into the bile. The main pathogenesis of cellular injury in WD is due to the presence of excess free copper which act as a catalyst for the Fenton reaction and help in creating free radicals. This led to oxidative stress and may subsequently contribute towards many diseases like Wilsons and neurodegenerative diseases (AD, PD, ALS, Prion disease). Elevated copper levels in the CSF samples of neurodevelopment disorder (NDD) patients give direct evidence of its association with regard to initiation and/or promotion of the diseases specially Alzheimer disease (AD) (8-9). Earlier research indicates that increased copper concentration may be as high as two folds in patients with neurological symptoms like dementia specially linked with AD (2, 10). On the other hand, recent studies have demonstrated that copper concentration rises with increasing age in the brain cortex. This leads to an overproduction of amyloid precursor protein (APP) and amyloid beta (aβ), which suppress cellular copper levels. If copper levels continue to rise, hypermetallation of aβ occurs that is also facilitated by mild acidosis. Even some forms of hypermetallated aß catalytically generate ROS/RNS. ROS includes Superoxide radical (O2.), Hydroxyl radical (OH.), RNS includes Nitrosyl (NO) and Peroxynitrile (9, 11-12). These ROS/RNS being highly reactive interact with biological molecules leading to alterations in cell function and Caspase dependent neuro-

nal death. On the other side ROS leads to DNA damage which triggers 8OHdG levels in brain (12-13).

Despite the abundance reports available regarding possible involvement of copper in NDD, the precise relationship among copper induced oxidative stress, development of memory impairment and development of neurodegenerative diseases are not fully elucidated. This study was designed to elucidate the effects of chronic exposure to copper in SD rat which were evaluated in terms of oxidative stress, and status of antioxidant defence system in brain and neurobehavioral parameters.

Materials and Methods

Animals

All animal experiment protocols were approved by the Institutional Animal Ethics Committee (IAEC). Male Sprague-Dawley rats (weighing about 80-100 g) were procured from the CSIR-Central Drug Research Institute, Lucknow, India and were kept in animal house of our institute. All animal husbandry procedures were maintained as per the Standard Operating Procedures (SOPs) followed in the test facility. All experimental animals were kept in standard polypropylene cages (3 rats /cage) and maintained under controlled room temperature (22 ± 2 °C) and humidity (55 ± 5 %) with automatically controlled cycle of 12 h light and 12 h dark. Standard laboratory animal feed and water (aqua pure) were provided *ad libitum*. The metal contents of the animal feed (in ppm dry wt.) were Cu 10, Mn 55, Co 5, Zn 45, and Fe 70. Animals were acclimatized to the experimental conditions for a period of 1 week prior to the beginning of the experiment.

Chemicals

Copper sulphate was obtained from Sigma-Aldrich (St. Louis, MO, USA), while all other chemicals were of "AnalaR" or "Extra pure" grade and obtained from Merck (Darmstadt, Germany) or Sigma (St. Louis, MO, USA).

Experimental design

Group 1st were received normal drinking water and served as a normal control group. Group 2nd and Group 3rd received copper sulphate ($CuSO_4 \cdot 5H_2O$) at the dose of 10mg/kg and 20mg/kg body weight, respectively via oral gavages. The dosing was done 0930 AM every day for 16 consecutive weeks. All animals were sacrificed under anesthesia after 16 weeks (Fig.1).

Blood and brain samples

After 16th week, exposure was stopped and rats were anesthetized under 50 mg/kg Pentobarbital. Blood samples were collected via intra-cardiac puncture, in centrifuge tubes and allowed to clot. Serum was collected by centrifugation at 7000g for 10 min and stored at 4°C. Animals were quickly dissected under anaesthesia, 24 h after the last dosing. Brain samples were removed, washed free of extraneous material with chilled normal saline and stored at -80°C until use for biochemical estimation.

Serum ceruloplasmin

Serum Ceruloplasmin was estimated spectrophotometrically by its oxidase activity with o-dianisidine dihydrochloride according to the method described by Schosinsky et al., (14). Serum samples were mixed with the optimal concentration of o-dianisidine dihydrochloride (7.88 mM) in 0.1 M acetate buffer (pH 5.0), and the absorbance of the final red solution was measured at 540 nm (14).

Element analysis

Copper estimation in brain was done by using of Atomic Absorption spectrophotometer. Brain tissues were digested using a conventional acid digestion method (Nitric acid and Perchloric acid) using Atomic Absorption spectrophotometer (15).

Neurobehavioral variables

Rota-rod test

Rotamax apparatus was used to evaluate the muscle strength and coordination in animal. Before the training sessions, the rat were habituated to stay on the stationary drum for 3 min. Habituation was repeated every day for 1 minute just before the session. In the final test period latency to falling was recorded automatically by photo-cells and the total latencies on the rod were analyzed (16).

Depression like behaviour in forced swim test

To evaluate the depression like symptoms we used glass cylinder height 40 cm, 30 cm in diameter which was filled with water to a height of 24 cm. The temperature of water was 25±1˚C. Measurement was carried out for five min and immobility time was measured with a stopwatch for the next five min (17).

Learning and memory in passive shock avoidance paradigm

The learning and memory capacities (acquisition and processing of information, decision making and response initiating) were assessed in copper exposed rats by passive avoidance in a shuttle box. The two-way shuttle box instrument was used for this test, which was composed of two adjacent Plexiglas boxes of identical

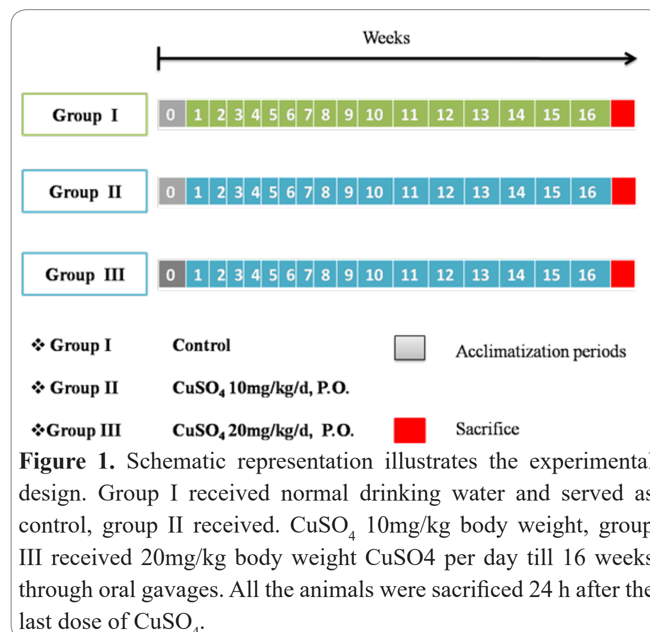

Figure 1. Schematic representation illustrates the experimental design. Group I received normal drinking water and served as control, group II received. $CuSO_4$ 10mg/kg body weight, group III received 20mg/kg body weight CuSO4 per day till 16 weeks through oral gavages. All the animals were sacrificed 24 h after the last dose of $CuSO_4$.

sizes (27 cm × 14.5 cm × 14 cm) with plexus floors. This methodology was completed in three successive days. On the first day, rats were acclimatized with the acquisition compartment. Each rodent was permitted a 10 min adjustment period with free access to either light or dark compartment of the avoidance training box after being placed in a shuttle box (to make familiar with tools). On the second day, rats were situated into the enlightened compartment and 30 sec later the door was elevated and latency of step-down was recorded as learning phase (initial latency). After entrance the dark compartment, the door was closed and when all four paws touched the grid, a low level electric shock (0.5 mA, 5 sec.) was applied. After 3 min, the rat was removed from the dark compartment and placed into the home cage. On day 3, in order to test short-term memory, the rats were set in the enlightened chamber once more, and 30 sec later the sliding entryway was raised, and latency of entering the dark compartment was recorded again, as step-through latency (STL). The cut-off time was 300 sec and no shock was delivered third day (18).

Locomotor activity

Spontaneous locomotor activity was evaluated by using optovarimax apparatus. The open-field behaviour of rats was assessed in a box measuring 90 cm X 90 cm X 30cm. sub-divided into 19 equal squares by black lines. A rat was placed in the centre of the open field; the movements of the rat were scored. Distance travelled was recorded in 10 minute duration (19).

Elevated plus maze

Elevated plus maze was used to evaluate Anxiety-like behaviour. The apparatus consists of two open arms (50.8×12 cm) and two closed arms (50.8 ×12 ×40.6 cm) that extended from a common central platform (10 ×10 cm), raised 72.4 cm above the floor. A camera (HD Logitech C525) was set on the apex of the maze. Rats were carried into test room in their home cages and were handled by the base of their tails at all the times. Animal were placed in the central square of the Plus-Maze facing an open and allowed them to explore the apparatus for 5 minutes. Number of arm entries and the time in each arm was recorded with the video camera and multiple stopwatches. After 5 minutes, rat were removed from the maze by the base of their tails and returned to their home cage. The maze was then cleaned with a solution of 70% ethyl alcohol and permitted to dry between tests. Length of time the animal spent in the open arms was recorded (20).

Novel object recognitions task

NORT test was performed in a top opened plastic red box (65 L x 65 B x 45 H cm). A camera (HD Logitech C525) was set on the apex of the box. The test procedure involved 3 different phases as, habituation phase, familiarization phase and recognition phase. Each animal was gently placed in the box without objects and allowed to explore for 5 min in 2 exploration sessions at 4 h interval during habituation phase. The familiarization phase was performed after 24 h of the first exploration session of habituation phase. Animals were familiarized with two identical wooden blocks over night. In the test session (timing < 10 min per rat) out of familiarized object one was replaced with novel one. Time spends with novel and familiar object was recorded. After the test session, objects were cleaned with 70% (vol/vol) ethanol to minimize any olfactory cues (21).

Biochemical variables

MDA levels

Malondialdehyde (MDA) considered as end product of lipid per oxidation, it was measurement in brain tissue by using thiobarbituric acid reactive substance method, according to Ohkawa et al., (22) with some modifications. In brief, the brain tissue were collected and rinsed with chilled PBS, then tissue was minced and homogenate was prepared in phosphate buffer (pH 7.4) containing EDTA (1 mM). The sample was centrifuged and the supernatant was used for the determination of MDA levels. The absorbance was measured at 532 nm. MDA levels was calculated from the standard curve using the 1, 1, 3, 3-tetramethoxy propane (97 %) and expressed as µM MDA/mg protein (22).

Reduced glutathione level

Reduced glutathione GSH(r) was estimated according to protocols described in Gupta et al., (2006) with some modification, the tissue homogenate separated as indicated above, equal volumes of 5 % sulfosalicylic acid were added and vortexed. The mixture was kept for 30 min in ice bath. After centrifugation, the supernatant was collected. GSH content was measured using Ellman's reagent 5, 5-dithiobis (2-nitrobenzoic acid) (DTNB) solution. GSH levels were calculated using a standard reference curve using reduced glutathione as a standard. Results were expressed in µM GSH/mg protein (23).

Nitrite contents

Measurement of Nitrite Content in the tissue homogenate was measured according to the method described in Giustarini et al., (24) with some modifications. For the determination of nitrite content, equal volumes of Griess reagent and supernatant were added in a 96- well plate and incubated for 10 min in dark with shaking, and then absorbance was measured at 540 nm. Nitrite levels were calculated using a standard curve using sodium nitrite as a standard and expressed as µM/mg protein (24).

Catalase activity

Ammonium molybdate forms a yellow complex with H_2O_2 and is suitable for measuring serum and tissue catalase activity. To analyze the catalase activity, incubate 0.2 ml of tissue homogenate with 1 ml 65 µM H_2O_2 in 6.0 mM sodium potassium phosphate buffer, pH 7.4 for 60 sec (sample 1). Prepare control reactions with 1 ml H2O2 plus 0.2 ml buffer (no enzyme control; blank 2) and 1.2 ml buffer (no enzyme/no substrate, blank 3). Stop the reaction by adding 1.0 ml of 32.4 mM ammonium molybdate to sample and control reactions. Determine the absorbance at 405 (25).

SOD activity

Superoxide dismutase (SOD) activity was estimated according to protocols described by Flora et al., (26). The reaction mixture was prepared which contained 1.2

ml of sodium pyrophosphate, 0.3 ml of PMS, 0.3 ml of NBT, 0.2 ml of supernatant, 0.8 ml of distilled water and 0.2 ml of NADH. The control reaction mixture was prepared and contained 1.2 ml of sodium pyrophosphate, 0.3 ml of PMS, 0.3 ml of NBT, 1ml of distilled water and 0.2 ml of NADH. Both mixtures were incubated at 37ºC for 90 sec and then reaction was stop with added 1 ml of acetic acid and the mixture was allowed to stand for 10 min. The absorbance was measured at 560nm (26).

Protein level

Supernatants (5 µL) of the brain tissue homogenate were incubated with solution D (2% sod. carbonate, 0.4% sod. hydroxide, 2% sod. tartrate, and 1% copper sulphate) for 10 min at 37 °C. Resulting solution was treated with Folin's reagent in 1:1 ratio for 30 min at 37 °C. Blue colour was developed. The absorbance was measured at 660 nm along with standard prepared with known concentration of BSA (27).

8-OHdG Level

Brain 8-OHdG Level was estimated by ELISA kit (MyBioSource San Diego USA) according to the manufacturer's instruction.

Statistical analyses

All results were expressed as the mean±standard error (SEM). Graph Pad (Prism 6) software were used for statistical analysis. Statistical differences in the variables between the groups were analyzed by one-way ANOVA followed by multiple comparisons with Tukey's test as well as regression analysis were performed along with correlation. The level of statistical significance was set at $p < 0.05$.

Results

Effect of copper exposure on body weight

Throughout the period of experiment, body weight of all animals in the control group increased with age. However in copper treated animal it showed a decrease but the values were not statistically significant (Fig.2).

Effect of copper exposure on tissue copper and Ceruloplasmin levels

Copper concentration increased in both copper exposed groups but it was observed that copper 20mg/kg treated group showed more pronounced uptake of copper levels in the brain tissues compared to the control group (Fig 3A). Ceruloplasmin level declined in both copper (10mg as well as 20mg/kg) exposed groups but statistically significant decrease was found in 20mg/kg treated group as compared to the normal control (Fig 3B).

Table 1. Effect of chronic copper sulphate exposure on serum ceruloplasmin level.

Parameters	Control	Cu 10 mg/kg	Cu 20 mg/kg
Ceruloplasmin activity (Unit/ml)	18±1.5	14±1.4	10±0.71**

All the values are expressed as mean± SEM (n = 5), **P < 0.01 vs. Control.

Effect of copper exposure on neurobehavioral parameters

Copper sulphate exposure decreased the passive avoidance response in animals to both copper exposed groups but more significant changes were observed in the 20 mg/kg treated group compared to control (Fig. 4A). Forced swim test revealed that copper administration significantly increased the immobility time compared to controls (Fig. 4B). Significant difference between control and copper intoxicated rats in total number of entries in open arms in Elevated plus maze and novel object exploration time were noted (Fig. 4C and 4D). Number of entries in open arm in copper treated group was significantly decreased compared to controls. Interestingly, copper intoxicated rats spend significantly less time to exploring the new object than the familiar one compared with control group.

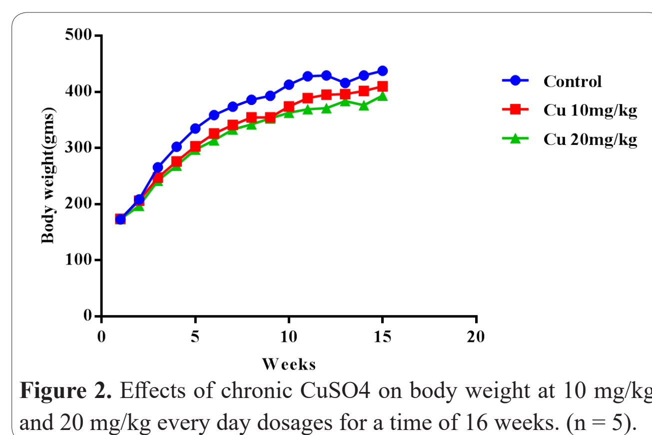

Figure 2. Effects of chronic CuSO4 on body weight at 10 mg/kg and 20 mg/kg every day dosages for a time of 16 weeks. (n = 5).

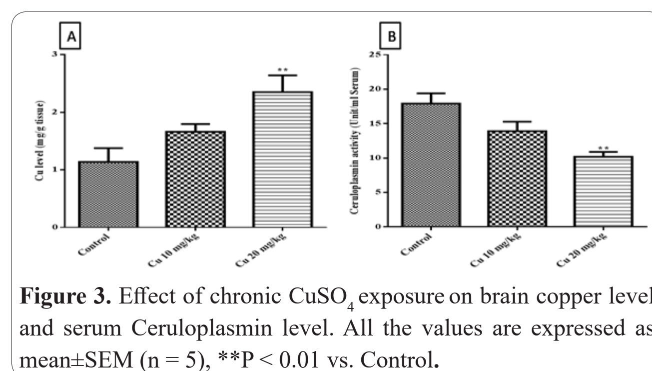

Figure 3. Effect of chronic $CuSO_4$ exposure on brain copper level and serum Ceruloplasmin level. All the values are expressed as mean±SEM (n = 5), **P < 0.01 vs. Control.

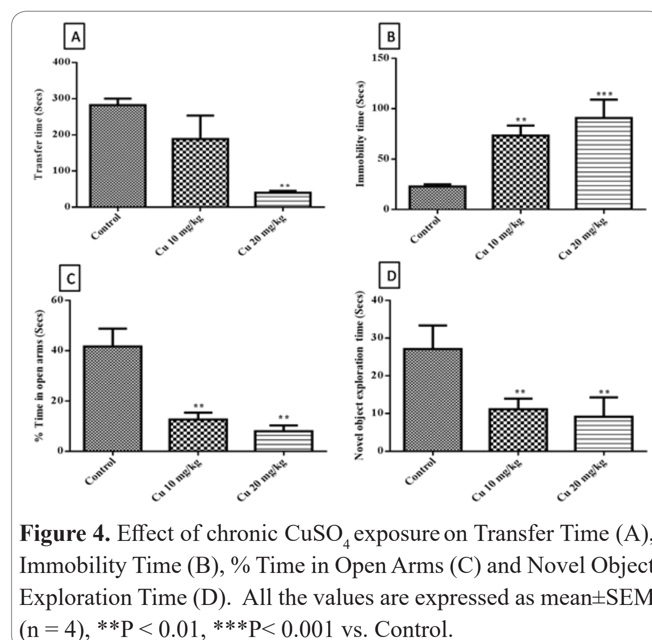

Figure 4. Effect of chronic $CuSO_4$ exposure on Transfer Time (A), Immobility Time (B), % Time in Open Arms (C) and Novel Object Exploration Time (D). All the values are expressed as mean±SEM (n = 4), **P < 0.01, ***P< 0.001 vs. Control.

Effect of expoure on the motor cordination and locomotors activity

The rotarod test results demonstrate a noticeable impairment in muscle strength and coordination in copper exposed rats (Fig. 5A). Obtained spontoneous locomotors activity results suggest that locomotors activity of the rat was signigcalty decresed in both copper treated groups in comparison to normal controls (Fig. 5B and 5C).

Effect of copper exposure on TBARS and 8-OHdG activity

TBARS level was significantly higher in both copper exposed groups (10mg and 20 mg/kg) compared to normal controls (Fig. 6B). 8-OHdG activity was significantly higher in 20 mg/kg copper exposed groups in the brain compared with the control group (Fig. 6B).

Effect of copper exposure on oxidative stress parameters

Level of reduced glutathione showed depletion in both copper sulphate exposed groups compared to normal control. However, both copper intoxicated animals (10 and 20 mg/kg) failed to induce statistically significant difference in rGSH level in the brain tissues as compared to the control (Fig. 7A). Copper sulphate exposure at the dose of 20mg/kg led to an increased nitrite level compared to the control group (Fig. 7B). It was also observed that Catalase and SOD activities were significantly decreased in the both copper sulphate treated groups compared to the control group (Fig. 7C, and 7D).

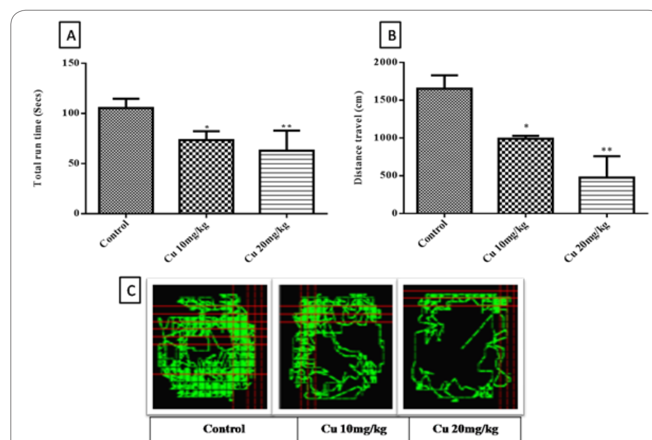

Figure 5. Effect of copper intoxication on the neuromuscular coordination (A) and locomotors activity (B&C) in rats using the Rotarod test and optovarimax apparatus. Values are the mean±SEM (n=4). *P<0.05, **P < 0.01 vs. Control.

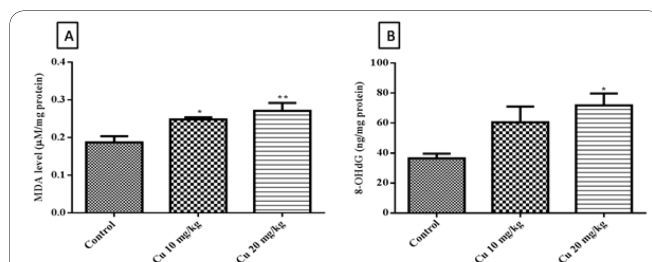

Figure 6. Effect of chronic $CuSO_4$ exposure on brain MDA level (A) and 8-OHdG activity (B). All the values are expressed as mean±SEM (n = 4), *P< 0.05, **P < 0.01 vs. Control.

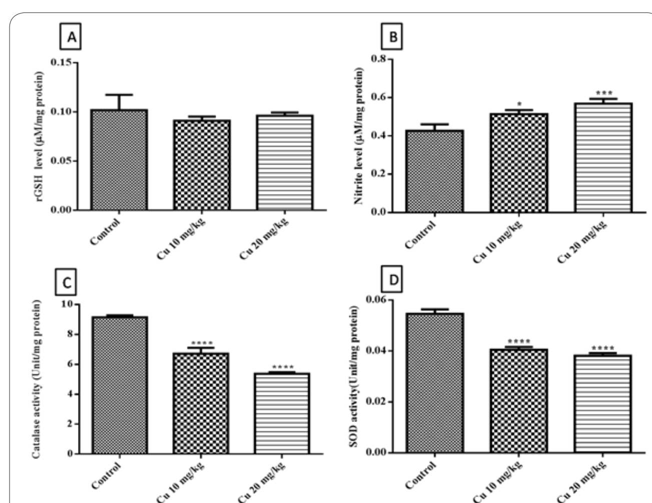

Figure 7. Effect of chronic $CuSO_4$ exposure on brain rGSH level (A), Nitrite level (B), Catalase Activity (C) and SOD activity. All the values are expressed as mean±SEM (n = 4), *P< 0.05, ***P < 0.001, ****P< 0.0001 vs. Control.

Discussion

Copper is one of the essential transition metal in the body, which act as a prosthetic group for variety of proteins and metalloenzymes, required for essential metabolic functions, cell homeostasis growth and development (28, 29). Copper exerts its action in the biological system by electron-transfer catalysis through freely exchangeable oxidation states Cu^{+1}(cuprous) and Cu^{+2} (cupric) (30). Copper has become the subject of vigorous research over numerous decades. Previous *in-vitro* as well as *in-vivo* a study robustly supports the involvement of excess copper with neurotoxicity and pathogenesis of various neurodegenerative diseases like AD, PD, and WD (31, 32). The brain and liver have been the major vulnerable organs for copper to exert its toxicological effects (33). The present study demonstrates that chronic dose dependent copper sulphate exposure in-

Table 2. Effect of chronic copper sulphate exposure on neurobehavioral parameters .

Parameters	Control	Cu 10mg/kg	Cu 20mg/kg
Passive avoidance test (transfer time in Seconds)	282±18	188±65	40±5.6**
Forced swim test (Immobility time in Seconds)	23±1.1	73±10**	91±9.2***
Elevated plus maze % Time spent in open arm (Seconds)	42±7.1	13±2.7**	8.0±2.3**
NORT (Novel object exploration time in Seconds)	27±3.1	11±2.8**	9.2±2.6**
Loco motor activity. Distance travel (cm)	1654±176	990±40*	476±163**
Rota-rod (Total run time in Seconds)	105.4±4.672	73.40±8.915*	62.98±9.986**
Passive avoidance test (transfer time in Seconds)	282±18	188±65	40±5.6**

All the values are expressed as mean± SEM (n = 5),*P< 0.05, **P < 0.01, ***P< 0.001 vs. Control.

duces oxidative stress in rat brain which is significantly correlated with changes in different neurobehavioral parameters. Our result shows that, there was reduction in body weight gain on exposure to copper sulphate at both 10 and 20 mg/kg doses. Decrease in the body weight on copper exposure might be attributed to the gastrointestinal (GI) disturbances and additionally due to hepatotoxic effects. In contrast, it has been earlier been reported that copper sulphate exposure decreases body weight (1200 ppm for 16 week) in male SD rats (34). Copper level results demonstrated a significant copper accumulation in brain compared to control group animals. It was earlier been reported that brain copper accumulation also leads to neurobehavioral changes (4). We also studied the effects of chronic copper exposure on, learning and memory, locomotion, muscle strength and co-ordination in SD rats. Neurobehavioral results unveil significant copper neurotoxicity as evident from open arm dwell time was reduced, increase immobility time, decreased locomotors activity, impairment in object recognition, impairment of passive avoidance retention and shorter latency to fall time in rota rod apparatus. The previous investigations also report that copper toxicity led to significant behavioural changes, which were related with higher copper concentration in the brain (4, 13, 33). We also observed that chronic administration of copper resulted in a striking impairment of muscle strength and motor coordination in animals, which is confirmed by the significant decline in retention time on the rota rod apparatus in both 10mg as well as 20mg/kg group animals. Our results are in concurrence with the past findings observed that chronic copper exposure caused a significantly decrease in the retention time on the rota rod apparatus (33). Chronic exposure of copper elicit depressive symptoms which were confirmed by the elevated plus maze and forced swim test Main molecular mechanism behind copper induce depression is still not clear but it was reported that copper level increased in the depression patient (35, 36). There were two possible mechanisms explaining the development of depression. i) Binding of copper ions changes prion protein conformation, that might be playing an important role in regulation of NMDA receptors and development of depression (37) and ii) Imbalance of zinc and copper levels may modulate GABA, eventually leading to a lower concentration of neurotransmitter in the brain and contribute to depression (38).

Our neurobehavioral experiments results demonstrated that chronic copper exposure triggered memory impairment which is confirmed by the passive avoidance test and novel object recognition task. Cognitive functions have been widely used as a sensitive marker of neurotoxicity (39). Recently it was reported that, copper accumulation in the hippocampus and striatum cause behavioural changes in toxic milk mice, an animal model of WD (40). To support our data it has been observed that copper exposure decreases AChE activity in the brain that might be the possible mechanism for copper induced memory impairment (33, 41). Furthermore it was reported that copper trigged precipitation of amyloid beta protein in the hippocampus region of the brain may be responsible for the memory impairment (1).

Ceruloplasmin is a primary copper-carrying protein in the circulatory system, it incorporates 95% of serum copper and additionally it plays an important role in iron metabolism. Ceruloplasmin synthesis mainly occurs in hepatocytes, and later on it is found in plasma. In our study chronic copper exposure showed decrease in the activity of serum ceruloplasmin. Ceruloplasmin level gets decreased because increased copper concentration reduces the Ceruloplasmin biosynthesis in liver (41, 42). It has been reported that oxidative/nitrosative stress emerged as one of the important factor that involve in the pathogenesis of several neurodegenerative diseases (43, 44). In a biological context ROS have pivotal roles in cell signalling and homeostasis (45). Chronic copper exposure produces a large amount of ROS/RNS resulting in damage of membrane lipids, proteins, and nucleic acids that may become irreversible, and may even cause cell death (46, 47). Hydroxydeoxyguanosine (8-OHdG) has been widely used as a biomarker for oxidative stress induced DNA damage that can be the outcome of ROS attack on cell (48, 43). There is however evidences that ROS production leads to 8-OHdG formation (49). We observed that 8-OHdG activity was increased in the brain tissue of both copper exposed groups but it increased more significantly in the 20 mg/kg copper exposed group. It was reported that the augment 8-OHdG activity in tissues may be an outcome of oxidative stress and over load of ROS/RNS. Earlier investigation has revealed that an elevated oxidative stress and 8-OHdG activity participate in the different neurodegenerative disorders like AD, PD, and WD (50, 51). To further strengthen the findings of copper sulphate is associated with oxidative stress generation. Our results indicate that dyshomeostasis of oxidant and antioxidant level in the brain tissue in the form of increased TBARS, nitrite, level and simultaneous decrease in antioxidant enzyme concentrations such as reduced GSH, catalase, SOD. In our finding TBARS level was significantly increased in both copper exposed groups (10 and 20mg/kg). Possible cause of copper elevated TBARS level could be attributed to the ROS generation which is main culprit of lipid per oxidation. These results are comparable to the earlier studies (52, 53) which stated that TBARS is critical markers of lipid per oxidation and it is considered as a major manifestation of oxidative stress. This hypothesis is further supported by one more study (32) which suggested that copper significantly raised TBARS formation. It was attributed to ROS production particularly involvements of hydrogen peroxide in the lipid per oxidation. Our results are also in agreement with an earlier study (54) suggesting that copper significantly increased lipid per oxidation levels in the hepatocytes through ROS generation which may lead to significant elevation in TBARS levels. Concurrently, copper overloads significantly decrease the Superoxide dismutase activity in both copper sulphate treated groups in the brain tissue. The reduction in Superoxide dismutase activity might be due to the excess formation of ROS in the brain. SOD is one of the well-known cellular antioxidant enzymes rapidly neutralizes supper oxide into H_2O_2, further breakdown by Catalase enzyme into water and oxygen (55). The effect of high copper burden on hepatic lipid peroxidation and antioxidation defence capacity by oral administration of high copper sulphate (500 mg/kg body weight) 5 days a week for 2

months suggest that excessive copper accumulation in the liver depleted SOD activity. The study concluded that reduced SOD activity may be due to an increased MDA concentration in serum and liver homogenates (48). Our results showed that catalase activity was significantly decreases in both copper exposed groups. Copper exposure increases the H_2O_2 production in the brain that might be one of the possible reasons of decline in catalase activity. A clinical study carried out by Ozturk et al., (56) observed that catalase activity was significantly low in patients with recurrent aphthous stomatitis (RAS) with elevated serum copper concentration (56). Additionally, Singh et al., (57) investigated that treatment of Copper Sulphate on *Channa punctatus* (Freshwater Fish) causes reduction in Catalase levels (57). It was observed reduced glutathione level was less in the both copper sulphate exposed rats, however it was not significantly decrease in comparison with control group. The reasons of decreased concentration of rGSH by copper stress remain unknown but might be partly attributed to the increase in ROS production that is caused by copper stress, which consumes large amounts of GSH (58). In the present study we have observed that copper exposure significantly increase nitric oxide (NO) levels, which is unique marker of the nitrosative stress possible cause of increase massive transcription of NOS-II gene in brain. Nitric oxide (NO) is known as an unstable signalling molecule that can be produced by three different NO synthase (NOS) isoforms. It plays a crucial role in a wide range of physiological processes in the body. For instance, in central nervous system (CNS) it acts as a neurotransmitter, while in cardiovascular system NO acts as a blood vessel relaxant. However, when generated in excess NO can be neurotoxic (59). To support our data Cuzzocrea et al., (60, 61) explored that nitric oxide concentration was extensively high in copper exposed rat and while treatment with inducible NOS (NOS-II) inhibitors amino guanidine significantly decreases the NO level (60).

The present results confirmed that over accumulation of copper in brain may lead to increased oxidative stress which might be responsible for neurobehavioral changes in rats. It can be concluded that copper exposure at a higher dose (20mg/kg) may lead to more significant toxicity in contrast to a comparative lower dose (10mg/kg). Our developed rat model is an effective model for assessment of copper toxicity which may be useful for understanding the pathomechanisms and therapeutic investigation of copper toxicity. In light of these outcomes and other past findings, it would be worthy to further investigate the precise molecular mechanism of copper induce neurodegeneration.

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Interest Conflict

The authors declare that they have no conflict of interest.

Authors' contribution

JK and KBS executed the experimental work, calculated the data and prepared the first draft. SJSF planned the experiment, and finalized the manuscript.

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