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Neurobehavioral and acetylcholine esterase study of brain after dietary copper deficiency in *Rattus norvegicus*

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ABSTRACT
Copper a quintessential element is required for normal brain development and functioning. Study investigates the effects of dietary copper deficiency on behavior of Wistar rats. Pre-pubertal rats were divided into four groups: (i) negative control (NC) fed standard feed (ii) copper control (CC) fed 126 nmol Cu/ gm diet (iii) pairfed (PF) fed 126 nmol Cu/gm diet but the diet given was based on the diet consumed by CD group the previous day and (iv) copper deficient (CD) fed 6.3 nmol Cu/ gm of diet for 2-, 4- and 6- weeks. Neurobehavioral tests - Rota rod treadmill, Morris water maze and open field were studied. Acetylcholine esterase activity was also assessed. Rota rod treadmill test revealed significant decrease in motor function in CD groups. Decrease was also evident when PF groups were compared with controls although after 2 weeks the decrease was non significant. Similar trend was evident in Morris water maze test wherein the spatial learning decreased significantly (P<0.05) in CD groups. In open field test, the CD animals showed anxious behavior evident by decreased tendency to explore the area. Acetylcholine esterase activity in brain sub-regions decreased (P<0.05) in CD groups. Decline in activity was observed in PF groups. Copper deficiency during growth period caused deleterious effect on brain as reflected in behavioral dysfunction such as impairment in motor function, memory/learning ability and suppressed exploratory behavior and decreased acetylcholine activity. This indicates that imbalance of copper if prolonged for a long duration would profoundly affect the physiology of brain as well as behavior.
Key words: *Copper deficiency, Neurobehavioral studies, Aceytlcholine*

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INTRODUCTION

From microbes to human, copper functions as an essential trace element [1]. Copper has high redox potential [2] which enables it to function as cofactor for proteins[3] involved in myriad of biological functions: iron uptake, signaling in eukaryotic organisms, myelin formation, erythropoeisis, synthesis of hormones, antioxidant protection, change of immune system, neurodevelopment, synaptogenesis, axon extension, change of neurotransmitter receptor activity and synaptic transmission [4; 5; 6; 7; 8; 9]
Approximately 100 mg copper is reported in human body [10] with 8.8 mg in brain [11] sufficient to perform the normal functioning including the central nervous system [12]. Highest concentration of copper in brain next to heart and liver has been reported [13]. Copper distribution appeared to be high in grey matter compared to that of white matter [14; 15]. Several authors [1; 16; 17] reported the presence of copper in basal ganglia, hippocampus, cerebellum, substantia nigra, hypothalamus, olfactory bulb, and locus coeruleus, numerous synaptic membranes, and in the cell bodies of cortical pyramidal and cerebellar granular neurons. Copper has the ability to cross the blood brain barrier and blood-cerebrospinal fluid barrier although protein bound copper cannot be transported [18].Copper homeostasis is also maintained by astrocytes as well as metallothionein [19; 20]. Copper may enter the brain through various copper transporters located at the brain barriers such as Cu transporter-1 (Ctr1,enters through endothelial cells of brain, transported to astrocytes and then to neurons, localized also in excitatory neurons), divalent metal transporter-1(DMT-1), ATP7A (released into brain parenchyma) and ATP7B [18; 21;22;23;24;25;]. Acetylcholine esterase activity is essential for terminating nerve impulses within the central nervous system and also for maintaining pulsatile cholinergic stimulation. Brain stem, cerebellum, nervous system (peripheral and autonomic), muscles and red blood cell membrane contains acetylcholine esterase which has specific roles [26]. With abundance of

copper concentration in brain sub regions the present study investigates the effects of copper deficiency on neurobehavioral and acetylcholine esterase activity after short duration of copper deficiency.

MATERIAL AND METHODS

Experimental Diet

Basal synthetic diet was prepared by using ICN Research Diet Protocol (1999) with slight modifications. The Synthetic basal diet was composed of: Egg white/ albumin (180 gm), Corn oil (100gm), Corn starch (443gm), Sucrose (200 gm), Cellulose (30gm), Choline chloride (2gm), DL-methionine (7gm), AIN-76 salt mixture (35 gm), AIN-76C vitamin-antibody mixture (10gm). The experimental diets differed only in copper content containing 126 nmol Cu/g and 6.3 nmol Cu/g by adding appropriate amount of copper sulphate which was confirmed by GBC 902 atomic absorption spectrophotometer at 324.8 nm in air acetylene flame.

Experimental Protocol

Prepubertal male Wistar rats (30-40 days; 35-50 gm) were divided into four groups (24 animals each for the 2-, 4- and 6- week). (a) Negative control (NC) fed standard rodent pellet diet (Ashirwad industries; Chandigarh, India), (b) Copper control (CC), (c) Pair fed (PF) both were fed 126 nmol Cu/ gm of diet but later group's diet was based on diet consumed by CD group the previous day to determine the starvation effect of reduced diet intake and stress effects of synthetic diet. The above three groups were provided tap water *ad libitum*. (d) Copper deficient (CD) fed 6.3 nmol Cu/ gm of diet and given demineralized water *ad-libitum*.

The experiment was carried out for 2-, 4- and 6- weeks. The study was complied according to the guidelines for the use and care of laboratory animals set by Committee for the Purpose of Control and Supervision of Experiments on Animals (No. 1678/GO/Re/S/12/CPCSEA dated 16.06.17) and approved by Departmental Animal Ethics Committee, University of Rajasthan, Jaipur, India.

Male Wistar rats were housed individually in polypropylene cages with stainless steel grills. Cages, grills and water bottles were washed with detergent solution, demineralized water and finally rinsed in 1% EDTA solution prepared in demineralized water so as to avoid contamination and subsequent removal of copper. After the completion of experiments animals were randomly selected from each group and subjected to various neurobehavioral studies. Few animals from each group were separated, cranium exposed and the whole brain was carefully removed. The brain was separated on a chilled glass plate resting over ice, into four sub-regions viz., frontal cortex, parietal, occipital and cerebellum and subjected for the estimation of acetylcholine esterase activity.

Neurobehavioral studies

(i)Rota Rod Test

Motor function was evaluated by the procedure described by [27]. A test trial began when the rat was placed on the rod facing the back of the chamber and ended when it fell to the floor, with time recorded. After training on a stationary rod and then on a rod rotating at the constant rate of 25 rpm and time (sec.) on rod (latency to fall, maximum 180 sec). 3 trials were run each day for 5 consecutive days

(ii)Morris water maze test

Spatial learning ability was assessed by the method described by [28] with slight modification. The water maze consisted of a round tank (150 cm in diameter, 50 cm deep) with water temperature being maintained at 25ºC. A platform (10 cm^2) submersed 2 cm under the water surface was placed on the center of one of the four quadrants of the tank and maintained in the same position during all trials. Several distal visual cues were placed on the walls of the water maze room. During the experiment, the scores for latency to escape to the platform were recorded. The training session consisted of four consecutive trials during which the animals were left in the tank facing the wall, and allowed to swim freely to the escape platform. If the animal did not find the platform in 120 sec it was gently guided to it. The animal was allowed to remain on the platform for 10 sec after escaping to it and was then removed from the tank for 20 sec before being placed at the next starting point in the tank. This procedure was repeated six times with different starting points. The test session was performed 48 h later and was similar to the training session, except that the number of trials was reduced to three.

(iii)Open field test

The open field test procedure given by [29]was used to monitor the general and exploratory behavior of rodents with slight modification The open field consisted of a black painted wooden box which measured 120 cm x 120cm x 50 cm (L x W x H). The floor was divided into 20 cm squares with white paint. At the beginning of each test, the animal was placed in the centre square of the field and given five minutes to explore the field freely. The rats were monitored directly by the 2 observers standing on opposite sides of the field to prevent the animal's inclination to either side and following parameters were analyzed.

1) Locomotion: Total no. of squares entered by the animal with all four paws.

2) Rearing frequency: Number of times rat stood on its hind legs.
3) Grooming frequency: Number of times the animal touched or rubbed its snout with its paws.
4) Anxious behavior: Number of entries into the 4 central most squares

The open field box was washed with 30% alcohol solution before placing the subsequent animals in it in order to avoid possible biasing effect due to odor clues left by previous rats.

Neurochemical study

Acetylcholine esterase

The acetylcholinesterase activity was measured following the method of [30]. 5,5-dithiobis-2 nitrobenzoic acid (DTNB- coloring reagent;0.01M dissolved in 1M phosphate buffer , pH7.0) and sodium bicarbonate, 0.1M phosphate buffer (pH 8.0) were prepared. Briefly, tissue sample was added to 3.0 ml pH 8.0 buffer, 20.0 µl substrate and 100.0 µl DTNB. The degradation of acetylthiocholine iodide was measured at 412 nm and the results are expressed as µmoles substrate hydrolyzed/min/mg protein.

Statistical analysis

All quantitative data were analyzed using the GraphPad Prism® software (version 7). The data were statistically analyzed with One way ANOVA followed by Post hoc (Tukey's multiple comparisons test) if found significant and were expressed as the mean (±) SEM. P< 0.05 was considered significant.

RESULTS

Copper deficient groups of 2-,4- and 6 weeks exhibited impairment with latency to fall being significant (P<0.05) reduced compared to their respective control groups (NC and CC) and PF groups. However, PF groups when compared with their respective NC and CC groups, a non-significant decrease was observed in 2 weeks but significant (P<0.05) decrease was observed in 4- and 6- weeks. Non-significant change in fall off time was recorded between NC and CC groups (Table 1).

In spatial and latent learning / memory test the copper deficient groups indicated marked effect on the navigation ability. The CD groups took more time to reach the platform in the water maze which was significant compared to their respective control groups (NC and CC) and PF groups after 2-, 4- and 6- weeks. However when PF groups were compared to their respective control groups, there was a non-significant change after 2- weeks but a significant reduction in performance was observed in 4- and 6- weeks PF groups (Table 1).

In open field test a significant (P<0.05) decrease in number of squares crossed, rearing frequency, decreased central area enteries as well as grooming frequency was recorded in copper deficient groups compared to their NC, CC and PF groups. After 2 weeks of copper deficiency experiment ,non significant change was when PF groups were compared to their respective controls (NC and CC) although decline was significant (P<0.05) in all tested parameters after 4- and 6- weeks (Table 1).

Table 1 –Behavioural performance of Wistar rats after 2-, 4- and 6- weeks of dietary copper deficiency (Mean±SEM)

Groups	Rotarod Performance (s)	Open field Test performance				Escape latency (s) in Morris water maze test
		Locomotion frequency	Rearing frequency	Grooming frequency	No. of entries in central region	
2 NC	80.83±0.74	21.33±0.88	13.16±0.87	9.5±0.56	9.33±0.66	75.00±0.81
2 CC	80.22±1.38	20.50±1.05	12.83±0.83	9.1±0.60	9.16±0.83	73.66±0.91
2 PF	79.83±1.30	20.33±0.76	12.66±0.71	8.66±0.33	9.00±0.36	76.16±1.01
2 CD	75.76±0.98^{c*e*f}	13.16±0.90^{c*e*f*}	7.5±0.76^{c*e*f*}	4.00±0.36^{c*e*f*}	5.66±0.33^{c*e*f*}	96.50±1.36^{c*e*f*}
4 NC	103±2.06	25.66±1.17	15.66±0.66	11.83±0.65	10.50±0.50	78.50±0.71
4 CC	100±1.23	25.16±0.47	14.83±0.83	11.50±0.76	10.33±0.55	77.66±1.22
4 PF	72.16±0.94^{b*d*}	17.83±1.13^{b*d*}	10.50±0.88^{b*d*}	6.66±0.33^{b*d*}	7.83±0.54^{b*d*}	84.00±1.12^{b*d*}
4 CD	64.83±1.35^{c*e*f*}	8.83±0.60^{c*e*f*}	5.66±0.55^{c*e*f*}	3.83±0.47^{c*e*f*}	3.83±0.60^{c*e*f*}	104.00±1.06^{c*e*f*}
6 NC	126.16±2.02	27.16±1.13	17.66±0.71	13.16±0.60	10.83±0.40	81.50±0.88
6 CC	125.33±1.66	26.83±0.94	16.33±0.80	13.00±0.57	10.66±0.66	80.00±0.85
6 PF	67.33.50±0.95^{b*d*}	15.66±0.61^{b*d*}	8.66±0.42^{b*d*}	5.66±0.61^{b*d*}	7.16±0.47^{b*d*}	93.16±1.27^{b*d*}
6 CD	56.00±1.01^{c*e*f*}	5.33±0.61^{c*e*f*}	5.16±0.47^{c*e*f*}	3.16±0.47^{c*e*f*}	2.16±0.47^{c*e*f}	111.00±1.18^{c*e*f*}

a= NC Vs CC, b= NC Vs PF, *p<0.05 Significant
c= NC Vs CD, d= CC Vs PF,
e= CC Vs CD, f= PF Vs CD
Multiple comparison procedures were performed for 2-, 4- and 6 weeks experimental groups.

A significant (P<0.05) decrease in acetyl cholinesterase activity was observed in all sub- regions of brain of dietary copper deficient groups compared to respective NC, CC and PF groups. A non-significant decline in the activity was observed when pair-fed groups were compared with NC and CC groups in

frontal, parietal and occipital regions whereas in cerebellum this decline was significant (P<0.05) after 4- and 6- weeks of copper deficiency (Table 2).

Table 2- Acetylcholine esterase activity (µmoles substrate hydrolyzed/min/mg protein) in brain sub- regions of Wistar rats after 2-, 4- and 6- weeks of dietary copper deficiency (Mean±SEM)

Groups	Acetylcholine esterase (µmoles substrate hydrolyzed/min/mg protein)			
	Frontal	Parietal	Occipital	Cerebellum
2 NC	1.7683±0.0107	1.963±0.0161	1.820±0.0178	2.3016±0.0319
2 CC	1.7783±0.0107	1.966±0.0171	1.823±0.0111	2.388±0.0923
2 PF	1.7666±0.0148	1.9616±0.0103	1.803±0.0143	2.305±0.0423
2 CD	1.536±0.0125^{c*e*f*}	1.626±0.0104^{c*e*f*}	1.71±0.0109^{c*e*f*}	1.913±0.0166^{c*e*f*}
4 NC	2.160±0.0101	2.26±0.0138	1.94±0.0164	3.608±0.0182
4 CC	2.163±0.0156	2.24±0.0199	1.93±0.0216	3.645±0.0232
4 PF	2.130±0.0172	2.2183±0.011	1.916±0.017	3.345±0.0128^{b*d*}
4 CD	1.463±0.0143^{c*e*f*}	1.533±0.0128^{c*e*f*}	1.623±0.0119^{c*e*f*}	1.618±0.01659^{c*e*f*}
6 NC	3.605±0.0143	3.165±0.0159	2.026±0.0143	5.12±0.0178
6 CC	3.61±0.0176	3.175±0.0173	2.025±0.0253	5.166±0.01534
6 PF	3.59±0.0125	3.165±0.0125	1.995±0.0125	5.06±0.01534^{b*d*}
6 CD	1.255±0.0143^{c*e*f*}	1.375±0.0160^{c*e*f*}	1.4366±0.012^{c*e*f*}	1.3133±0.0249^{c*e*f*}

a= NC Vs CC, b= NC Vs PF, *p<0.05 Significant
c= NC Vs CD, d= CC Vs PF,
e= CC Vs CD, f= PF Vs CD
Multiple comparison procedures were performed for 2-, 4- and 6 weeks experimental groups.

DISCUSSION

Copper deficiency although has been rare but now it is more widespread and may become more prevalent [31]. Numerous factors are responsible for decrease in bioavailability of copper [32;33].In the present study copper deficient groups (2-, 4- and 6- weeks) adversely affected the motor coordination, neuromuscular strength spatial learning / memory further decreasing the exploratory behavior of animal. Altered motor dysfunction accounted to change in cerebellar function after copper deficiency [34] with marked changes in size of Purkinje cells and distinct irregularities in Purkinje cell monolayer [35] was reported. Abnormalities were seen in humans after copper deficiency evident by posterior column dysfunction, distal sensory loss accounting for disabled gait ataxia - condition resembling amyotrophic lateral sclerosis (ALS) [36]. ALS due to mutation in gene encoding Cu-Zn superoxide dismutase (SOD1) exhibited marked loss of motor neurons in motor cortex, brain stem and spinal cord [37].The mechanism by which copper deficiency causes neurologic dysfunction is unknown. Reduced dopamine concentration was reported after dietary copper deficiency [38].Contrary to this, dopamine b-monooxygenase mRNA concentration was reported to be high in medulla oblongata in 22 day old copper deficient female but not male rats when comparison was carried out with their respective controls [39] although decreased norepinephrine concentrations but normal dopamine levels in brain after dietary as well as genetic copper deficiency was also recorded [40]. Dopamine and noradrenaline are crucial neuromodulators controlling brain states, vigilance, action, reward, learning, and memory processes [41]. Decreased myelin formation, synaptogenesis [42], oligodendrocytic protein expression [43] after copper deficiency was observed. Dietary copper deficiency indicated motor deficits as observed by rotarod test. Gingrich [44] showed that nervous system controls the anxiety behavior with GABAergic and serotoninergic systems playing a significant role in its regulation. Several authors [45] accounted impairment of nervous system due to oxidative damage which can lead not only to depression but also to high anxiety levels and anxiety disorders. A key role in neuronal resistance towards oxidative stress is played by Nrf2 [46] with Cu^{2+} having a significant role in Nrf2 activation [47]. Authors [48] observed that individuals exhibiting anxious behavior face difficulty not only to concentrate on a particular task but also cannot efficiently process relevant information leading to decline in cognitive performance as well as motor control. Loss of memory (working and long-term declarative) was observed in Alzheimer's disease and can be correlated with parameters of structural / functional brain integrity [49]. Decline in locomotion, rearing, grooming behavior with enhanced anxiogenic behavior, memory /learning ability in copper deficient rats indicates changes in brain due to low copper level. Due to perturbed copper homeostasis dysfunction neurobehavioral changes were observed in the present study.

The acetylcholine esterase activity in present study is found to be significantly reduced in all brain sub regions studied. The decrease in AChE activity can be correlated with a decrease in choline

acetyltransferase activity, free radical generation and oxidative stress. Our findings are also supported by several authors [50] Moreover, Kazi and Oommen[51] observed that oxidative stress if prolonged for longer duration can lead to neurotoxicity. The subsequent generation of OH radical further inhibits AChE activity in rat brain as well as human recombinant AChE activity [52; 53; 54]. The altered concentration of AChE can probably be due to enhanced oxidative stress generated by dietary copper deficiency .The increased oxidative stress after copper deficiency is supported by impairment of erythrocyte membrane affecting morphology, osmotic fragility, deregulation of hematopoiesis and altered the hematological profile [55] as well as functional aspect of testes (glutathione, SOD, Cu-Zn SOD, Mn SOD and catalase) [56].

CONCLUSION

Dietary copper deficiency in prepuberatal period affected acetylcholine esterase activity due to enhanced oxidative stress which may possibly be one of the factor responsible for exacerbating neurodegeneration. Concomitantly neurobehavioral deficit was evident by altered motor function, spatial learning /memory and exploratory and anxiety behavior. This implicates a significant role of copper in neurochemical as well as neurobehavior during critical growth period.

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