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ORIGINAL ARTICLE

Effect of Copper on L-Cysteine/L-Cystine Influx in Normal Human Erythrocytes and Erythrocytes of Wilson's Disease

Nabarun Mandal[1] · Debojyoti Bhattacharjee[2] · Jayanta Kumar Rout[3] · Anindya Dasgupta[4] · Gorachand Bhattacharya[5] · Chandan Sarkar[3] · Prasanta Kumar Gangopadhyaya[6]

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Abstract Wilson's disease is a disease of abnormal copper metabolism in which free serum copper level is raised. The objective of the study was to determine, whether in Wilson disease, L-cysteine/L-cystine influx into RBC was decreased or not and the specific amino acid transporter affected by copper in normal human RBC. For L-cysteine/L-cystine influx, ten untreated cases, ten treated cases and ten age and sex matched healthy controls were recruited. To study the effect of copper on L-cysteine/L-cystine influx in RBC, 15 healthy subjects were selected. RBC GSH and L-cysteine/L-cystine influx were estimated by Beautler's and Yildiz's method respectively. In untreated cases, L-cysteine/L-cystine influx and erythrocyte GSH level were decreased showing that elevated level of free copper in serum or media decreased L-cysteine/L-cystine influx in human RBC. Copper treatment inhibited L amino acid transporter in normal RBC specifically.

Keywords Human erythrocyte · L-Cysteine/L-cystine influx · Reduced glutathione · Wilson's disease

Introduction

Wilson's disease is an autosomal recessive disease affecting ATP7B gene [1]. In this disease copper is not excreted in the bile and it accumulates in brain, kidney, liver and RBC resulting in copper toxicosis [2]. The frequency of Wilson's disease is about 1 in 30,000-40,000. The frequency of carriers of ATP7B gene's mutation is about 1 %. An affected patient's sibling has 1 in 4 risks [3]. There are more than 200 mutations known [4]. The disease may present with hepatic (at early/late teens); neuropsychiatric manifestations (typically in early twenties), or with hemolytic anaemia [3]. In Wilson disease, serum ceruloplasmin level is low (90 % of cases) [5], 24 h urinary copper excretion is elevated (usually >100 μg/day) [6] along with increased copper level per gram of dry weight of liver tissue (>250 μg/gm) [7]. Though total serum copper level decreases as copper is mostly bound to ceruloplasmin, there is increased "free non-ceruloplasmin copper" in serum as well as in tissues [8].

✉ Debojyoti Bhattacharjee
debojyoti1979@rediffmail.com

Nabarun Mandal
dr.nabarunmandal@gmail.com

Jayanta Kumar Rout
dr.jayantarout@yahoo.in

Anindya Dasgupta
anindya11@yahoo.com

Gorachand Bhattacharya
drgbhattacharya@gmail.com

Chandan Sarkar
csarkar2347@rediffmail.com

Prasanta Kumar Gangopadhyaya
pkganguly100@yahoo.com

[1] Department of Biochemistry, Midnapore Medical College, Paschim Medinipur, WB 721101, India

[2] Department of Biochemistry, Murshidabad Medical College, Berhampore, Murshidabad, WB 703482, India

[3] Department of Biochemistry, R.G.Kar Medical College, Kolkata, WB 700004, India

[4] Department of Biochemistry, Calcutta National Medical College, Kolkata, WB 700014, India

[5] Department of Biochemistry, MGM Medical College, Kishanganj, Bihar 855107, India

[6] Department of Neurology, KPC Medical College, Kolkata, WB 700032, India

Pathogenesis is due to oxidant tissue injury induced by free copper [3]. Increased free radical production by elevated plasma free copper through transition metal catalyzed Fenton reaction leads to oxidative stress induced injury, resulting in altered metabolism and severely compromised antioxidant status in RBC, hepatocytes and other tissues, particularly in acute phases characterized by hemolysis [9].

Previous studies showed that presence of excess copper ions led to the lower level of GSH concentration and G-6PD activity in erythrocytes [10, 11]. There is decrease in other RBC antioxidant parameters like superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, resulting in increased lipid peroxidation. Similarly, sulfhydryl groups and α- tocopherol showed the significant decline in their study. To combat free radical injury, GSH along with other enzymes and antioxidants is necessary [12]. Synthesis of GSH is dependent upon supply of L-cysteine [13]. The rate limiting step of GSH Synthesis is incorporation of L-cysteine to L-glutamate to from γ glutamyL-cysteine [14].

Almost all plasma cysteine exists as cystine. Cystine, the oxidized form of cysteine is transported in the cells via sodium independent transporter [15]. In human RBC, L-cysteine is transported by both specific sodium dependent (ASC System) and nonspecific but large capacity sodium independent system (L-transporter). L-transporter system is less susceptible to inhibition by competing substrates at plasma levels [16].

Keeping this background in mind, the aim of the current study is to find out whether, in Wilson disease, L-cysteine/L-cystine influx in RBC is decreased by high concentration of copper or not and the specific receptor responsible for this phenomenon.

Materials and Methods

Heparinized blood (10 U/ml) was collected aseptically from antecubital veins from each subject. The protocol was approved by institutional ethics committee of Calcutta National Medical College. Proper informed consent was taken from every participant. The blood was centrifuged at 4 °C for 5 min at 2555×*g*, and the plasma, and buffy coat were discarded. RBC was washed thrice with ice cold 0.9 % normal saline and was made to 50 % hematocrit in phosphate buffered saline (pH 7.4, prepared by mixing equal volume of 150 mM sodium phosphate buffer, pH 7.4 and 0.9 %, NaCl) containing 10 mM D-glucose and was used for studying L-cysteine transport (both through ASC system & Transporter L) & reduced glutathione level in RBC. As in our study there was no way to distinguish between L-cysteine influx and L-cystine influx in RBC through transporter L, we assigned the process as L-cysteine/L-cystine influx.

Plasma fasting glucose, bilirubin (total and direct), total protein, albumin, aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase estimation were performed on Erba-Mannheim, XL-600 autoanalyser. Total count of RBC, PCV (Packed cell volume), MCV (mean corpuscular volume) and MCHC (mean corpuscular hemoglobin Concentration) were estimated using automated cell counter (Sysmax, KX-21).

Serum ceruloplasmin was measured by Ravin's method [17]. Serum copper and 24 h urinary copper were measured by the method of Abe et al. [18] using the color reagent 4(3, 5-dibromo-2 pyridylazo) N-ethyl-N-sulfopropylaniline. Erythrocyte GSH was estimated using the method of Beutler et al. [19]. Erythrocyte L-cysteine influx was measured by the method of Yildiz et al. using 10 mM L-cysteine hydrochloride [20]. This concentration of L-cysteine hydrochloride was also used by Rizvi and Maurya [13] and Kumar and Maurya [21]. RBC L-cysteine influx was expressed as μM/gm Hb/Hr. For copper induced inhibition of L-cysteine/L-cystine influx, RBC suspension (final concentration25 %) was incubated initially with 20 μM copper for 2 h prior to L-cysteine/L-cystine influx in erythrocytes. Hemoglobin was estimated by cyanmethemoglobin method [22]. Erythrocyte GSH was also expressed in terms of μM/gm Hb.

Statistical Analysis

The results were expressed as mean ± SD across the three study groups. Comparison for significance of difference between their mean values was done by analysis of variance (ANOVA) test using the Statistical Package for Social Sciences (SPSS) version 16 for windows programme. Multiple linear regression analyses were performed to show predictive values of serum ceruloplasmin, serum copper levels and RBC L-cysteine/L-cystine uptake on RBC GSH levels in both treated and untreated cases as well as in controls. p value <0.05 was considered to be statistically significant.

Results

Table 1 shows a summary of data obtained from controls, new case group and treated case groups of Wilson disease with regard to basic biochemical alterations. Multiple comparisons showing differences between different groups regarding GSH concentration in RBC and the L-cysteine/L-cystine uptake rate by RBC

Table 1 Inter group comparison of physical and clinical parameters

Parameters	Mean ± SD			*p** value		
	Controls	New cases	Treated cases	Controls versus new cases	Controls versus treated cases	New cases versus treated cases
Age (years)	20.8 ± 7.97	19.8 ± 8.77	20.6 ± 6.62	>0.05	>0.05	>0.05
Sex (male/female)	5/5	4/6	6/4			
Serum ceruloplasmin (mg/dL)	26.77 ± 5.95	11.89 ± 3.5	13.6 ± 3.40	<0.05	<0.05	>0.05
Serum copper μg/dL	91.23 ± 11.19	48.33 ± 8.84	40.63 ± 6.11	<0.05	<0.05	>0.05
Urinary copper (μg/day)	50.44 ± 22.92	329.7 ± 136.81	142.8 ± 47.23	<0.05	<0.05	>0.05

n = 10 in each group

Sig. value (*p* value) <0.05 is taken as significant

Table 2 Multiple comparisons by analysis of variance (ANOVA) with Bonferroni correction showing difference between control, new case and treated case groups regarding GSH concentration in RBC and rate of L-cysteine/L-cystine uptake by RBC

Dependent variable	Controls (0)	New cases (1)	Treated cases (2)	Mean Difference (I–J)	Sig. (*p* value)
RBC GSH concentration (μmol/g of Hb)	8.51 ± 0.76	5.08 ± 0.62	8.08 ± 0.88	2.99600 (0–1)	<0.001
				0.43200 (0–2)	0.641
				−3.42800 (1–2)	<0.001
RBC L-cysteine uptake rate (μmol/g of Hb/hour)	11.26 ± 1.50	6.58 ± 0.74	10.32 ± 0.72	4.67900 (0–1)	<0.001
				0.93900 (0–2)	0.169
				−3.74000 (1–2)	<0.001

0 = controls; 1 = new cases; 2 = treated case; n = 10 in each group

Sig. value (*p* value) <0.05 is taken as significant

Table 3 Multiple linear regression analysis to show predictive value of serum ceruloplasmin, RBC cysteine/cystine uptake rate and serum copper levels on RBC glutathione concentration in control, new case and treated case group

Constant	Controls Sig. (*p* value)	New Cases Sig. (*p* value)	Treated Cases Sig. (*p* value)
Ceruloplasmin	0.751	0.545	0.766
Serum copper	0.196	0.457	0.651
L-Cysteine uptake rate	0.081	0.041*	0.596

Dependent variable: GSH concentration in RBC; n = 10 in each group

* Sig. value (*p* value) <0.05 is taken as significant

demonstrated that GSH concentration in RBC and L-cysteine/L-cystine uptake by RBC in new case group was significantly lower ($p < 0.001$) in comparison to treated case group of Wilson disease and control group. However, there was no statistically significant difference existed between treated cases and control groups in these respects (Table 2). Multiple linear regression analyses done to show the predictive values of serum ceruloplasmin, RBC L-cysteine/L-cystine uptake and serum copper levels on the RBC GSH concentration in new case groups demonstrated that the GSH concentration in RBC is significantly dependent on L-cysteine/L-cystine uptake in RBC ($p < 0.05$) but not on serum copper concentration and serum ceruloplasmin concentration (Table 3).

In this study, it was also observed that in treated cases and control groups the GSH concentration in RBC was not significantly dependent on L-cysteine/L-cystine uptake rate in RBC, serum copper concentration and serum ceruloplasmin concentration (Table 3).

Discussion

Experimental models performed by us showed the effect of negative effect of high copper (20 μM) level upon RBC L-cysteine/L-cystine uptake of healthy human individuals. It was also found that the L-transporter sysyem is primarily responsible for L-cysteine/L-cystine uptake in RBC compared to the ACS system. By altering buffer composition, it has been shown that it is the L-transporter (but not the ASC system) that is affected by copper (20 μM) treatment. While studying the mechanism of action of the copper inhibition of L-cysteine/L-cystine uptake by L-transporter, we found in the literature that both in vitro and in vivo, CuSO4 reduced the activity of 12 O tetradecanoylphorbol 13 acetate (TPA) stimulated protein kinase C suggesting inhibition of protein kinase C may be due to free copper ions [23]. Since L-transporter activity is activated by protein kinase C [24], copper inhibition of L-transporter may be responsible for inhibition of L-cysteine/L-cystine uptake via L-transporter in presence of copper (20 μM).

In vitro, extracellular cystine at 1.0 mM sustained GSH synthesis in GSH depleted RBCs only at the rate of 20 % of the maximum rate obtained with L-cysteine [25]. This finding suggests that cysteine is a better candidate for GSH synthesis than cystine. The concentration of L-cysteine in plasma is in about 10 μM [26]. Since cysteine is transported in the RBCs also by high capacity, low affinity L-transport system, L-transporter would be expected to have considerable effect at the higher concentration of amino acid used experimentally. Nonphysiological extracellular concentration of L-cysteine estimates accumulation capacity of this amino acid in intact human RBC. Closs et al. [27] used this approach. They obtained surprising data on the accumulation capacity of murine cationic amino acid transporters (mCAT—1, mCAT—2, mCAT—2a) in Xenopus laevis oocytes at a nonphysiological extracellular concentration of 10 mM L-arginine. Incubation of oocytes in 10 mM arginine for 6 h led to accumulation of arginine to the level of 0.6, 1.4 and 6 folds in mCAT—1, mCAT—2 and mCAT—2a expressing oocytes respectively.

Serum free copper level is high in Wilson's disease. The normal free copper value of serum is 1.6-2.4 μ mol/l and in untreated disease, it is often very high as 7.9 μmol/l [3]. Furthermore, the increased copper level in the liver cells appears to inhibit the binding of copper to apoceruloplasmin and leads to low level of ceruloplasmin in plasma [2]. This high free copper produces inhibition of L-cysteine uptake by L-transporter of RBC in Wilson disease. To the best of our knowledge, this is the first report of diminished L-cysteine influx in human RBC in Wilson disease. Also as RBC GSH level was low, decreased uptake of L-cysteine in human erythrocytes may affect intracellular synthesis of GSH. Rizvi and Maurya [13] had concluded that since glutathione synthesis in erythrocytes is dependent on the availability of L-cysteine, the decreased influx of L-cysteine in human erythrocytes during human aging may be a factor contributing to low GSH concentration. Similar phenomenon may occur in human RBCs in Wilson disease. This can further aggravate oxidative stress induced by Fenton reaction. That RBC GSH level is also dependent upon L-cysteine/L-cystine uptake was also evident from multiple linear regression in untreated patients of Wilson disease (Table 3).

Reduced glutathione concentration as well as rate of L-cysteine/L-cystine uptake in erythrocytes, improved significantly in treated cases in comparison to new cases. There were no significant differences regarding these parameters between treated cases and controls.

The dependency of erythrocyte GSH concentration upon the L-cysteine/L-cystine uptake was no more present in treated cases. This was probably because after treatment, the regeneration of GSH was restored and consumption of GSH by free radicals and copper ions was reduced along with improved L-cysteine uptake.

In conclusion, 20 μM copper inhibits L-cysteine/L-cystine uptake in human RBC by inhibiting the amino acid transporter L. In Wilson disease, free non-ceruloplasmin serum copper level is high which inhibits the L-cysteine/L-cystine uptake in RBC of untreated patients of Wilson disease. Also in the untreated patients of Wilson disease, GSH concentration in RBC is low and is significantly dependent on the L-cysteine/L-cystine uptake in RBC.

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Author's Contribution Dr. Nabarun Mandal and Dr. Debojyoti Bhattacharjee developed the study protocol and were involved in patient screening and enrolement. Dr. Gorachand Bhattacharya conceptualised the study and participated in the development of the project. Dr. Chandan Sarkar and Dr. Jayanta Kumar Rout were involved in the measurement of parameters. Dr. Anindya Dasgupta contributed to data analysis. Dr. Prasanta Kumar Gangopadhyaya participated as an expert in diagnosis, treatment and follow up of cases.

Compliance with Ethical Standards

Conflict of interest All the authors involved in this study declares that they have no conflict of interest.

Ethical Approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed Consent Informed consent was obtained from all individual participants included in the study.

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