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Original Article

Correlation of cobalt binding activity of albumin with the common markers of oxidative stress in thalassemia syndrome patients

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Key Words
Albumin; Cobalt binding; Protein carbonylation; Thalassemia syndrome; Sialic acid; Sialoprotein

Abstract
Scanty evidence exists for change in cobalt binding activity of albumin in Thalassemia. The aim of the present study was to observe whether cobalt binding activity of albumin would be altered in thalassemia patients receiving chronic transfusion and also whether the same would be correlated with oxidative stress markers. The present hospital-based, non-interventional, cross-sectional case control study was undertaken in Medical College and Hospital, Kolkata, India. Sixty patients with thalassemia syndrome requiring blood or erythrocyte transfusion for more than five years were randomly selected as cases. Age and sex matched controls were selected from apparently healthy individuals who were neither thalassemic trait nor carrier. Cobalt binding activity of albumin (ACB), thiobarbituric acid reactive substances (TBARS), protein carbonyls, protein bound sialic acid/sialoprotein ratio were measured in serum samples. There was no significant difference in cobalt binding activity between case and control. Serum protein carbonyls and TBARS were significantly higher in case population compared to control group. Ratio of serum protein bound sialic acid to serum mucoprotein was significantly lower in thalassemia cases. The study demonstrated that ACB was not correlated with serum TBARS, serum protein carbonyls as well as protein bound sialic acid/sialoprotein ratio in thalassemia. In conclusion, ACB is not superior to other markers of oxidative stress in identifying oxidative stress in thalassemia patients receiving repeated transfusions.

INTRODUCTION

The diagnostic albumin cobalt (Co^{2+}) binding (ACB) test is based on the observation that the affinity of serum albumin for Co^{2+} is reduced after N-terminus modifications possibly resulting from oxidative stress [1, 2]. Indeed there are reports of a reduction in ACB in diseases associated with sustained oxidative stress [3, 4]. An increase in oxidative stress has been observed in thalassemia patients receiving chronic transfusion [5-9]. However scanty literature exists regarding the relation of ACB to oxidative stress markers in these patients.

The present study had been undertaken to observe whether cobalt binding activity of albumin would be altered in thalassemia patients receiving repeated transfusion for more than five years and also whether it would be correlated with the established markers of oxidative stress like serum thiobarbituric acid reactive substances (TBARS) and level of protein carbonyl groups. This study also endeavoured to assess whether any alteration of serum protein bound sialic acid/serum mucoprotein (seromucoid) ratio takes place in thalassemia.

MATERIALS AND METHODS

Study design

The present study was a hospital-based, non-interventional, cross-sectional case control study. This work was undertaken in the Department of Biochemistry of Medical College and Hospital, Kolkata, India in collaboration with the Institute of Hematology & Transfusion Medicine (IHTM).

Subjects

60 cases (mean age was 24.56 ± 10.75 years including 25 women) with thalassemia syndrome were randomly selected from the patients attending thalassemia clinic in IHTM. Demographic data of these cases indicated haemoglobin E (HbE) beta thalassemia, diagnosed by high pressure liquid chromatography and subsequent molecular characterization; their pretransfusion hematocrit and ferritin levels were recorded as to be 0.201 ± 0.039 and 2360 ± 923 µg/l, respectively. Further, all patients had significant hepatosplenomegaly, 14 out of the 60 patients had undergone splenectomy and had received repeated blood or erythrocyte transfusion (at 3 to 4 week intervals) for a period of more than 5 years. Repeated blood transfusion for such a long period can exclude non-transfusion dependent (NTDT) patients.

Clinical history and relevant data were collected from patient's files with prior permission of the attending physician. The time from last transfusion to the time of sampling was at least thirty days. The sampling was done immediately before the next transfusion. All the patients belonged to the same socioeconomic group and hence it may be assumed that their dietary habits were more or less similar. All of them were on calcium and folic acid supplementation in equivalent dosage. None of the patients or control subjects enrolled in this study received antioxidant supplementations or vitamin E that could affect the results. None of the cases were on iron chelation therapy as they could not afford any form of iron chelation.

The age and sex matched 60 controls (mean age = 24.63 ± 10.69 years including 23 women) were randomly selected from apparently healthy individuals who were neither thalassemic trait nor carrier [10]. None had any history of blood transfusion, anemia, infection and any acute or chronic disease state. All the cases and controls were informed about the purpose of the study and written consent for inclusion in the study and for the publication of the study report was obtained. The study was approved by the Institutional Ethics Committee.

Experimental procedure

10 ml venous blood was collected from the median anticubital vein of the subjects and the collected clotted blood was centrifuged at 1500 rpm speed for 3-5 min. All the tests were done with serum harvested from clotted blood.

The cobalt binding activity of serum albumin was measured by the method described by Christenson *et al* [4] and modified in Biochemistry Department of Medical College and Hospital. Intra-assay coefficient of variation (CV) was 4.7% (0.0151/0.32); CV was calculated by measuring ACB of 20 samples, dividing the standard deviation by mean value and expressed in percentage.

The TBARS level of the serum samples was determined by the method described by Dahle *et al* [11], intra-run CV was 5.14% (0.384/7.47)]. The carbonylated protein level was measured by Levine *et al*'s [12] method with some modifications; intra-assay CV was 4.85% (0.051/1.05). Measurement of serum protein bound sialic acid was done by modified Aminoff's method [13]; iIntra-assay CV was 5.72% (0.165/2.88)]. Serum mucoprotein was measured according to Winzler *et al* [14]; intra-assay CV was 4.22% (3.11/73.67). Finally, serum protein and albumin were measured by the biuret and bromocresol green dye-binding method, respectively, using the XL-600 Autoanalyzer (Transasia; Mumbai, India).

Data analysis

Data analysis was performed using the SPSS software (version 17.0; Chicago, IL, USA). Data were normally distributed (Chi-square test). Statistically significant difference was determined by the Student's t-test. All P values are 2-sided, with values less than 0.05 considered significant. Correlation coefficients were calculated according to the Brave-Pearson function. The four parameters measured were examined whether they exhibit any bivariate and partial correlation.

RESULTS

Table 1 displays all the results of the two groups. Results are displayed in the form of mean ± standard deviation (standard error). It displays the results of unpaired t-test for equality of means of the control population and diagnosed cases of thalassemia who received chronic blood transfusion. It is evident that there is no significant difference in means of serum ACB between case and control group ($P = 0.287$). Serum TBARS was significantly higher in diagnosed cases (with multiple blood transfusions) than controls ($P < 0.001$). Serum protein carbonyl group was significantly higher in thalassemia cases compared to controls ($P < 0.001$). Serum protein bound sialic acid/serum mucoprotein rate was significantly lower in thalassemia *vs* control cases ($P < 0.001$). Serum protein bound sialic acid was significantly lower in thalassemia cases *vs* controls ($P < 0.001$).

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Table 1. Serum albumin cobalt binding activity (ACB), thiobarbituric acid reactive substances (TBARS), protein carbonyl, protein bound sialic acid levels, and protein bound sialic acid/mucoprotein rate[mean ± SD (SEM)]

Serum parameters	Thalassemia cases (n = 60)	Controls (n = 60)	t-value	P value
Albumin cobalt binding activity (U/ml)	23.89 ± 4.954 (0.64)	23.07 ± 3.222 (0.416)	-1.07	0.287
TBARS (μM/l)	12.895 ± 0.9542 (0.1232)	8.015 ± 0.7933 (0.1024)	-30.461	< 0.001
Protein carbonyl (nmol/mg protein)	2.1897 ± 0.18966 (0.02449)	1.0203 ± 0.23344 (0.0304)	-30.114	< 0.001
Protein bound sialic acid (μg/mg protein)	1.63 ± 0.5150 (0.066)	3.03 ± 0.44 (0.057)	15.914	< 0.001
Sialic acid/mucoprotein	0.0223 ± 0.00721 (0.00093)	0.0432 ± 0.00706 (0.00091)	16.087	< 0.001

SD, standard deviation; SEM, standard error of the mean.

Table 2. Bivariate correlation analysis among different parameters of oxidative stress in thalassemic (cases) and healthy individuals (controls)

		r_{12}	r_{13}	r_{14}	r_{15}	r_{23}	r_{24}	r_{25}	r_{34}	r_{35}	r_{54}
Cases (n = 60)	**r**	0.052	-0.070	-0.062	-0.081	0.522**	-0.595**	-0.573**	-0.272	-0.345**	0.893**
	P	0.691	0.597	0.636	0.540	**<0.001**	**<0.001**	**<0.001**	0.036	0.007	**<0.001**
Controls (n = 60)	**r**	0.352**	0.274	-0.169	-0.155	0.289	-0.082	0.044	-0.227	-0.179	0.895**
	P	0.006	0.034	0.198	0.237	0.025	0.533	0.739	0.081	0.171	**<0.001**

P, level of significance; r, Pearson's correlation; ******correlation is significant (2-tailed).

Table 3. Partial correlations of cobalt binding activity with others one eliminating the effect of remaining two parameters in cases and controls

	Control variables	Parameters with which ACB is correlated	Pearson's correlation (r)	Level of significance (P)
Cases (n=60)	X_3 and X_4	TBARS	0.069 (r_{12})	0.605
	X_2 and X_4	Protein carbonyl	-0.112 (r_{13})	0.403
	X_2 and X_3	Sialic acid/mucoprotein	-0.033 (r_{14})	0.808
Controls (n=60)	X_3 and X_4	TBARS	0.296 (r_{12})	0.024
	X_2 and X_4	Protein carbonyl	0.166 (r_{13})	0.214
	X_2 and X_3	Sialic acid/mucoprotein	-0.114 (r_{14})	0.396

X_2 = serum TBARS (μM/l), X_3 = serum protein carbonyl (nmol/mg protein), X_4 = sialic acid/mucoprotein ratio.

Table 2 shows the results of bivariate correlation analysis. There was significant positive correlation between serum TBARS and serum protein carbonyls as well as between serum protein bound sialic acid/mucoprotein ratio and serum protein bound sialic acid both in thalassemia cases and controls. Significant negative correlation also existed between serum protein TBARS and protein bound sialic acid/mucoprotein ratio in thalassemia patients whereas significant negative correlation was observed between protein carbonyls and protein bound sialic acid. ACB was not significantly correlated with serum TBARS (r = 0.052, P = 0.691), protein carbonyls (r = -0.07, P = 0.597) as well as serum sialic acid/sialoprotein (r = -0.062, P = 0.636) in the thalassemic patients.

Table 3 displays partial correlation of cobalt binding activity with one parameter of oxidative stress keeping the other parameters as fixed variables. It is evident that serum ACB is not significantly correlated with other parameters in thalassemia patients, whereas in controls ACB is correlated with TBARS when protein carbonyl groups and sialic acid/mucoprotein rate remain fixed.

DISCUSSION

It has been described by different workers that oxidative stress is increased in thalassemia syndrome [7, 15, 16], further aggravated by chronic blood transfusion resulting in accumulation of iron [5, 6]. Trombetta *et al* [9] have demonstrated significantly higher levels of protein carbonyl groups in the blood of thalassemia patients. Kassab-Chekir *et al* [17] have reported that serum TBARS concentration increased among thalassemia patients with iron overload. The observation of the present study corroborates with this finding in that the common markers of oxidative stress, i.e. TBARS and protein carbonyl groups, were higher in thalassemia patients as compared to the controls.

A significantly lower ratio of serum protein bound sialic acid to serum mucoprotein in thalassemia cases indicated increased removal of terminal sialic acid moiety in these patients. That this desialylation of serum protein may be a result of increased oxidative stress can be inferred from observation of several other studies [18-20]. Rajendiran *et al* [18] have shown that

higher levels of protein carbonyls and lower levels of serum protein bound sialic acid were found in dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) compared with dengue fever (DF) due to increased oxidative stress in DHF and DSS than in DF. Goswami *et al* [19] also showed that in hyperthyroidism, there was increased oxidative stress causing desialylation of protein. Thus it may not be inappropriate to infer from the observations of the present study that there was significant oxidative stress in the thalassemia patients receiving chronic transfusion.

A significant finding of the present study was that ACB was not significantly higher in thalassemia cases when compared to controls. Further, it was noted that though control data showed that cobalt binding activity was correlated with protein carbonyls and TBARS value obtained, bivariate analysis revealed that cobalt binding activity was not significantly correlated with serum TBARS, serum protein carbonyl groups as well as serum sialic acid/sialoprotein ratio in the thalassemia patients (Table 2). Even after test for partial correlation (keeping two variables fixed), ACB was not significantly correlated with marker of oxidative stress. This finding that ACB was not significantly higher in cases as compared to controls and further had no significant correlation with either protein carbonyls or TBARS in thalassemic individuals may stand in apparent contradiction to observations by several workers. Kountana *et al* [21] have described that ACB as measured by serum ischemia modified albumin (IMA) levels appears to represent a useful tool for excluding unstable angina in patients presenting to the emergency department with acute chest pain.

The results from a randomized control study determined a high level of IMA in testicular torsion of mature male Wistar rats, indicating its potential value in diagnosis of testicular torsion [22]. However, they failed to observe any significant correlation between ACB and TBARS levels, which is in agreement with the observation of the present study. Further, Awadallah *et al* [23] have observed increased levels of IMA in thalassemia patients. They have explained this observation to be a result of iron-induced oxidative stress and have highlighted the potential significance of IMA as a new marker of oxidative stress in such patients.

The finding of these studies may serve to highlight the role of oxidative stress in alteration of ACB probably resulting from structural modifications of circulating albumin in serum as described by Kountana *et al* [21]. The apparent contradiction in the finding of the present study could be discussed in this context; it may be suggested that although the ongoing oxidative stress in chronically transfused thalassemia patients was responsible for significantly higher protein carbonyl groups, TBARS and desialylation, it was insufficient for bringing about significant alteration in ACB. Also, it has been documented that alteration in ACB as a result of iron-induced oxidative stress appears to be primarily due to non-transferrin bound iron (NTBI). Since NTBI was not measured in the present study, it may be surmised that the rise in NTBI were not sufficient enough to alter the ACB. Future studies along this line incorporating a measurement of NTBI may serve to clarify this point. Further, it could be stated that oxidative stress may not be solely responsible for altered ACB. Another important cause has been described to be ischemia-reperfusion injury. Reperfusion of ischemic myocardium not only restores the blood supply but also causes production of free radicals, resulting in imbalance between oxidative and anti-oxidative process. Decrease in ACB has been documented to occur minutes after transient occlusion and reperfusion of a coronary artery during angioplasty [24]. Moreover, ACB returns toward baseline within 6 h suggesting that a modification had occurred. Reduced ACB also occurs in patients with spontaneous coronary ischemia, with an abnormal concentration detectable before demonstrable increase of cardiac troponin [3]. Since ischemia-reperfusion injury may be considered to be a significant component of some of these disease processes such as acute myocardial infarction, rheumatoid arthritis, *etc* it may be presumed to have a less definitive effect on the pathogenesis of some other diseases *viz* thalassemia. In this regard, the probability of altered ACB due to ischemia-reperfusion injury may be significantly higher in the former than in thalassemia.

A study conducted by Ozbek *et al* [25] revealed no statistically significant difference in ACB between diabetic metabolic syndrome and control group; however, serum total oxidant status levels were significantly higher in patients with diabetic metabolic syndrome than in control subjects. These findings are in agreement to the observations of the present work. From these observations, it may be suggested that significant alteration or reduction in ACB occurred predominantly during periods of injury or stress and that this alteration subsided in the post acute or chronic forms of the illness, though oxidation mediated tissue damage continued throughout the chronic phase. Therefore, it may not be entirely surprising that the present study did not indicate ACB as a predictor for oxidative stress in thalassemia syndrome with repeated blood transfusion and no correlation was established between cobalt binding activity and common markers of oxidative stress in thalassemia.

The present study however suffers from some limitations such as its small sample size and the heterogenous nature of the patient population being

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studied. Further, it would have been better to assess the NTBI and to find out the correlation between ACB and NTBI in thalassemia syndrome. Thus it may be concluded that the reduction in ACB occurred during periods of predominantly acute stress. This alteration subsided in the post acute or chronic forms of the illness like in thalassemia, though oxidation mediated tissue damage continued throughout the chronic phase.

COMPETING INTERESTS

The authors declare that they have no conflict of interests.

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