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Original Research Article

Evaluation of Serum Alkaline Phosphatase and Calcium as Emerging Biomarkers Regarding Calcium Homeostasis among Beta Thalassemia Major Patients in Tripura: North East India: A Cross-Sectional Study

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Abstract:

Background: Beta-thalassemia itself is an inherited disorder associated with beta-globin production deficiency. In this case affected children often require an urgent need of regular blood transfusion often results in secondary bone-related complications and iron overload. Regarding bone turnover, calcium itself is an important factor. Among thalassemia patients, their levels are typically below.

Methods: A cross-sectional study has been conducted among 65 thalassemia major patients of 5 to 15 years of age. The study duration was from September 2018 to July 2019. The main target was to estimate the levels of serum alkaline phosphatase (ALP) and calcium among patients with beta-thalassemia major and their correlative action regarding the frequency and duration of transfusion itself.

Results: There is a high incidence of hypocalcemia (98%) in beta-thalassemia patients. The calcium levels are decreased with association of significant rise in frequency and duration of transfusion. The parameters and transfusion did not show any significant correlation with serum levels of calcium. Levels of ALP were seen to be within normal limits in most of the patients.

Conclusions: Since the aforementioned study shows prolonged transfusion as one of the criteria in the patients associated with beta-thalassemia, estimation of levels of calcium should be regularly checked among these subjects, and steps to improve homeostasis of calcium may also be required to avoid various complications.

Keywords: Blood Transfusion, Alkaline Phosphatase, Calcium, Liver Dysfunction And Beta-Thalassemia Major.

Introduction

β (beta) thalassemia major is considered to be a bunch of genetic disorders which is an autosomal recessive in nature, results from a quantitative reduction of β-globin chain of hemoglobin (Hb). This can be an outcome of point mutations, or β-globin gene (HBB) deletions, or both. There is the normal synthesis of α-globin, which results in accumulation of excessive α-globin chains among erythroid precursors.

The output of this condition is ineffective erythropoiesis of RBCs that are prone to hemolysis. The β-thalassemia trait is occurred at rates of 1.4% to 3.4% in Central India, while β-thalassemia major (β-TM) recorded at 0.94%. Subjects with β-thalassemia major are associated with state of chronic anemia that needs frequent blood transfusion to reduce complications and increment of quality of life. [1] Iron overload is considered to be one of the major complications of multiple blood transfusions that often results in deposition of iron in several tissues, which is termed as hemosiderosis, in pancreas, liver, heart and endocrine glands (eg. Thyroid, parathyroid). [2] Thalassemia bone disease is considered to be a major significant barrier that often reflected as bone deformities, osteoporosis and overall growth retardation. The hidden or dormant cause of this is the dysregulation of remodelling of bone and altered calcium homeostasis that often associated with transfusion-induced hemosiderosis of parathyroid gland and liver. [3]

Calcium is considered as a mineral which serves as an important element of bone shape and structure, which provides rigidity with strength of teeth and bones. Besides this, calcium also represents itself as a signaling molecule required in various cellular activities, including muscular contraction, enzyme activity, and blood clotting and neurotransmitter release. [4]

The metabolism of calcium is hindered because of iron overload in thalassemia. On the other hand, Alkaline Phosphatase acts as an enzyme which is present with significant levels in kidney, bone, liver, intestine and placenta. Pre-β ALP is required for mineralization and bone formation, α1 ALP and α2 ALP is required for bile acids and lipids metabolism, χ-ALP is needed for absorption and digestion of various nutrients. In thalassemia, levels of ALP are fluctuated by liver dysfunction, bone deformities and treatment effects. Another globular protein is ferritin which acts as a storage of primary iron. Serum ferritin level portrays an important outcome into iron metabolism in thalassemia. Iron overload, chronic disease, inflammation and infection are usually associated with elevated ferritin level as a marker and acute phase reactant itself.

Ali et al. conducted a study that projected a low total serum calcium level among thalassemia patients and it was significantly lower as compared to the healthy group. [5] Sultana et al. conducted another study which defines that the transfusion dependent beta thalassemia patients possess high alkaline phosphatase level and low calcium levels.6 Yet, the paucity of data about multiple blood transfusion effects upon serum levels of alkaline phosphatase and calcium in β-thalassemia patients exists.

The aforementioned study is expressed to address this gap of information by estimating the serum levels of alkaline phosphatase and calcium as calcium homeostasis biomarker and tries to project an association between them along with duration and number of blood transfusion among β-thalassemia major patients.

Material and Methods

The aforementioned cross-sectional study has been performed at the Department of Biochemistry in collaboration with Department of Pediatrics of Tripura Medical College, Agartala, and Tripura. This study consists of a minimum of 65 subjects who were diagnosed with Beta Thalassemia minor, of the age between 5 to 15 years, who were visiting either in patient or outpatient departments. The duration of the study is from September 2018 to July 2019, after obtaining the approval from the ethical committee which is absolutely mandatory.

The initial aim was to analyze and measure serum levels of alkaline phosphatase, calcium and ferritin in relation of number and duration of blood transfusion received by the subjects.

Inclusion Criteria

It consists of all Thalassemia Major diagnosed cases, where verbal ascent from the children age of 7 to 15 years in presence of their parents, accompanied by written informed consent of the parents before conducting the study. While only written informed consent of the parents was taken for the children age group of 5 to 6 years. This study includes both male and female subjects.

Exclusion Criteria

All the Thalassemia minor subjects were excluded, along with other types of hemolytic anemia, like G6PD deficiency, sickle cell disease and hereditary spherocytosis. Patients with bone diseases like osteogenic bone cancer, osteomalacia, rickets, bone fractures and Paget's disease were excluded. Diseases of liver like intra and extrahepatic obstruction, cirrhosis and hepatitis were also excluded. Other chronic situations like CKD, endocrine diseases like hyperparathyroidism and hyperthyroidism were also grounds for exclusion in this study.

Around 5 ml of venous blood were collected under strict aseptic conditions by disposable syringes and put into clot activator tubes, then made it stand for 30 minutes in room temperature. The samples were then gone for centrifugation for 10 minutes at 3000 rpm, and then the serum was collected by a clean micropipette and stored at -20 degrees Celsius for further experiments.

Estimation of serum calcium is done by a colorimetric reaction with Arsenazo III, which was through spectrophotometric analysis. While Alkaline Phosphatase (ALP) levels were quantified by DGKC and SCE method with enzyme kinetics through ELITech clinical system Selectra Analyzers. A Chemiluminescent immunoassay (CLIA) was used for ferritin measurement.

Statistical analysis

These data were collected and entered in Microsoft Excel, and statistical analysis was done in Graph Pad Prism Software version 10.2.3 (347). To estimate the data distribution, the Shapiro-Wilk test and Kolmogorov-Smirnov test were performed for clarification of data as non-parametric or parametric. Non-parametric data were projected as inter-quartile ranges (IQR) and medians, while parametric data were projected as means and standard deviations (SD).

The student's Unpaired t test was performed for two independent groups in parametric comparisons, and one-way ANOVA was conducted for more than two independent groups comparisons, The Mann-Whitney U test was performed for two independent groups non-parametric comparisons, and the Kruskal-Wallis test was performed for more than two independent groups comparisons, While post-hoc analysis was conducted by using uncorrected Dunn's test, if required. Pearson correlation test was performed for correlational perimetric data analysis, and non-parametric data

analysis were done by Spearman rank correlation test. For all test, a p-value of <0.05 was considered to be statistically significant.

Results:

Demographic Profile

Table 1: ALP and Calcium levels status in patient's serum

Serum levels	Number of patents (n=65)	Percentage	Mean concentration
Calcium			
• Hypocalcemia (<8.7 mg/dl)	64	98%	7.28
• Normal calcium levels (8.7-10.7 mg/dl)	1	2%	Mg/dl
ALP			
• Below limit (<30 U/L)	5	8%	62.77
• Within limit (30-300 U/L)	60	92%	U/L

98% or 64 patients out of 65 were observed to possess serum calcium levels <8.7 mg/dl (Hypocalcemia), while only 2% or 1 subject had normal serum level of calcium. The mean concentration of calcium of serum in our subjects was 7.28 mg/dl. 92% or 60 subjects out of total 65 were observed to have serum ALP levels in normal limit for their age (30-300 IU/L), while 8% or 5 subjects had ALP levels of serum below the lower nd this study was 62.77 U/L.

Concentration Comparison of serum ALP and Calcium among male and female subjects.

Table 2: Median, Mean, SD, IQR and P value for serum ALP and Calcium across both gender.

Gender	Male (n=45)	Female (n=20)	p-value
o	Median 7.5(IQR 6.44-8.39)	7.51(IQR 6.55-8.02)	0.78
ALP parameters	Mean (SD) (61.67±23.82)	(65.25±25.35)	0.58

In our study, 69% or 45 patients were male and 31% or 20 patients were females among all 65 β-thalassemia patients.

There was no significant difference in serum ALP levels between males (M=61.67, SD=23.82) and females (M=65.25, SD=25.35); t(63)=-0.55, p=0.58 and serum calcium concentrations between males(M=7.5) and females(M=7.51); p=0.78.

Comparison of serum ALP of Calcium in different age groups: 48% or 31 patients were in the 5-10 years age group, and 52% or 34 patients were in 11-15 years age group.

Table 3: Median, IQR, Mean, SD and p-value for serum Calcium and ALP in both of the age groups.

Age groups	5-10 years (n=31)	11-15 years(n=34)	p-value
Calcium parameters (mg/dl)	Median(IQR) 7.5(5.96-8.19)	Median(IQR) 7.51(6.58-8.38)	0.64
ALP parameters (U/L)	Mean(SD) 64.55±27.754	Mean(SD) 61.15±20.65	0.57

Mann-Whitney U test -Non-parametric test, was conducted to compare the mean ranks of those parameters among the two age groups.

There is no significant difference in the concentration of serum calcium between age group of 5-10 years (Median=7.5) patients and of age group of 11-15 years (Median=7.51) patients; p=0.64.

Unpaired t-test-parametric test, was conducted to compare the serum ALP mean level between two age groups. There is no significant difference in serum ALP levels between subjects of age group 5 to 10 years (M=64.55, SD=27.75) and 11 to 15 years (M=61.15, SD=20.65); t(63)=0.56, p=0.57.

Comparison among concentrations of serum ALP and Calcium levels according to the duration of blood transfusion among β-thalassemia patients.

All the 65 subjects in our study were divided based upon their duration of BT into 3 groups; 1) 1 to 5 years, 2) 6 to 10 years and 3) more than 10 years.

Table 4: Median, IQR, Mean, SD and p-value of serum ALP and Calcium throughout the 3 groups based upon duration of BT in years

Duration of BT	1-5 years (n=19)	6-10 years (n=25)	>10 years (n=21)	p-value
Calcium parameters (mg/dl)	Median (IQR) 7.66(5.89-8.19)	Median (IQR) 7.5(6.5-8.37)	Median (IQR) 7.49(6.53-8.18)	0.99
ALP (U/L)	Mean (SD) 66.74±30.1	Mean (SD) 60.44±19.26	Mean (SD) 61.95±24.18	0.69

There was no significant difference among serum calcium concentrations between the groups; 2(2) = 0.03, p=0.99 and a median serum calcium concentration of 7.66 mg/dl in patients with 1 to 5 years of blood transfusion, 7.5 mg/dl for the patients with 6 to 10 years of blood transfusion, and 7.49 mg/dl for the subjects with more than 10 years of blood transfusion. One way ANOVA-parametric test, was conducted to compare the mean levels of ALP in the three groups. No statistically significant difference was observed in ALP levels between these groups, F (2.62) = 0.377, P=0.69.

Comparative estimation of concentration of serum ALP and Calcium levels along with number of blood transfusion in the β-Thalassemia patients.

Table 5: Mean, Median, SD, IQR and p-value for serum Calcium across the 3 groups based upon total number of blood transfusion

Number of BT	<100 BT (n=15)	100-200 BT (n=27)	>200 BT (n=23)	p-value
Calcium (mg/dl)	Median (IQR) 7.5(5.96-8.16)	Median (IQR) 7.8(6.46-8.4)	Median (IQR) 7.38(6.46-8.06)	0.78

No statistically significant difference been observed in serum concentration of calcium among the different groups, 2(2)=0.489, p=0.78.

Association between serum ALP and Calcium concentration with duration of blood transfusion in β-Thalassemia subjects.

Test for normally (normal distribution) has been performed for data received for this study for ALP and Calcium concentration using Shapiro-Wilk test and Kolmogorov-Smirnov test for every group. The attachment has been checked by using Pearson correlation test for parametric data, and association has been checked by using Spearman correlation test for non-parametric data.

Strength of association depicted by the r-value and expressed as following:-

- <0.3 – poorly correlated
- 0.3 to 0.59 – Fairly correlated
- 0.6 to 0.79 – Moderately correlated
- 0.8 and above – Strongly correlated

Table 6: Association among serum ALP and Calcium concentration along with duration of BT in β-Thalassemia patients

Duration of BT	1-5 years (n=19)	1-5 years (n=19)	6-10 years (n=25)	6-10 years (n=25)	>10 years (n=21)	>10 years (n=21)	Total (n=65)	Total (n=65)
Parameters	R-value	p-value	R-value	p-value	R-value	p-value	R-value	p-value
Calcium (mg/dl)	-0.12	0.63	+0.02	0.93	-0.58	0.005	-0.006	0.63
ALP (U/L)	-0.22	0.37	+0.10	0.62	-0.30	0.19	-0.11	0.38

Here the subjects with β-thalassemia undergoing blood transfusion were got to have statistically significant, poor negative correlation r(63)=-0.006, p=0.63, among their serum calcium concentration and poor negative correlation r(63)=-0.11, p=0.38, between their serum ALP level with the BT duration in years.

Table 7: Association between serum ALP and Calcium concentration with number of BT among β-Thalassemia subjects

Number of BT	<100 BT (n=15)	<100 BT (n=15)	100-200 BT (n=27)	100-200 BT (n=27)	>200 BT (n=23)	>200 BT (n=23)	Total (n=65)	Total (n=65)
Parameters	r- value	p- value	r- value	p- value	r- value	p- value	r- value	p- value
Calcium	0.17	0.54	-0.50	0.008	-0.25	0.26	-0.14	0.27
ALP	-0.09	0.76	-0.34	0.08	-0.07	0.76	-0.15	0.25

Here subjects with β-Thalassemia undergoing blood transfusion were seen to have statistically insignificant, poor negative correlation r(63)=-0.14, p=0.27, among their serum calcium concentration and poor negative correlation r(63)=-0.15, p=0.25 among their serum ALP. Level and the total amount of blood transfusion they underwent.

Comparison of the concentration of serum ALP and Calcium among patients with serum ferritin concentration of <1000ng/ml vs >1000ng/ml.

Non- parametric test,-Mann-Whitney U test, was conducted for comparison of the median of these parameters between two groups.

Table 8: IQR, Median and p-value for serum ALP and Calcium among subjects with serum ferritin concentration of , <1000 ng/ml vs >1000ng/ml.

Serum ferritin	<1000 ng/ml (n=8)	>1000 ng/ml	p-value
Parameters	Median (IQR)	Median (IQR)	
Calcium (mg/dl)	7.10 (6.00-8.14)	7.53 (6.46-8.37)	0.42
ALP (U/L)	56.50 (47.25-84.50)	60.0 (46.0-79.0)	0.87

No significant difference been observed in serum ALP concentration (p=0.87) and serum calcium concentration (p=0.42) among patients with serum ferritin concentration of <1000 ng/ml when compared to patients with serum ferritin concentration of >1000ng/ml.

Discussion

β- Thalassemia major itself can be referred as an autosomal recessive disorder, which is hereditary and hematological in nature, often characterized by mutations in the β-globin gene (HBB), that leads to absence or deficiency of active beta-globin protein. [7] The disorder leads to faulty erythropoiesis featured as a chronic state of anemia which needs lifelong blood transfusion.

Several research works have depicted that there is an alteration of calcium homeostasis among patients with β-thalassemia major, which leads to high incidence of hypocalcemia among patients with β-Thalassemia when compared to normal healthy individuals. [5] Yet there is limited information about the serum calcium levels association with number and duration of blood transfusions among β-Thalassemia subjects. [9]

In this study that included 65 subjects of β-thalassemia major, 98% or 64 patients were assessed to have calcium levels of >8.7 mg/dl, depicted as hypocalcemia, while only 2% or 1 patient had normal serum calcium levels. 7.28 mg/dl is the mean concentration of serum calcium in our study. These outcomes are similar to the studies conducted by Sultan et al [10], Ali et al [8], and Sultana et al [6], which showed hypocalcemia in 66%, 44.73% and 67.5% among β-thalassemia patients respectively, associated with mean serum calcium concentration of 8.1,7.9 and 6.9 mg/dl respectively. It projects that serum concentration of calcium is decreased among majority of the β-Thalassemia subjects.

There is no significant difference (p= 0.58 and 0.78 for ALP and Calcium respectively) between concentration of serum ALP and calcium between male and female. We have compared the concentrations of serum ALP and Calcium between two groups of different ages, 5-10 years and 11-15 years. However, no significant difference was seen in the concentration of serum calcium levels between subjects of age group of 5-10 years (Median=7.5) and subjects of age group of 11-15 years (Median=7.51); p=0.64. There is also no significant difference in serum ALP levels among subjects of age groups of 5-10 years (M=64.55, SD=27.75) and of 11-15 years (M=61.15, SD=20.65); P=0.57.

Upon observation of serum ALP and Calcium levels among subjects who had undergone blood transfusion for 1 to 5 years, 6 to 10 years and >10 years, we have found that there was no statistically significant difference among serum calcium concentrations between these different groups, p=0.99 associated with a median serum concentration of 7.66 mg/dl for subjects of 1 to 5 years of BT, 7.5 mg/dl for the subjects with more than 10 years of BT and there was no statistically significant deviations of ALP levels between these groups, p=0.69. Meanwhile, there was no statistically significant difference between ALP concentration (p=0.87) and calcium concentration (p=0.42) among patients with serum ferritin concentration of <1000 ng/ml as. Compared to subjects with serum ferritin concentration of >1000 ng/ml.

Conclusion

After thorough analysis of the outcomes of the study, we can presume that the subjects with β-thalassemia major associated with multiple blood transfusion (BT) have high association of hypocalcemia. There was complete absence of any significant correlation between serum calcium levels and number or duration of BT within the initial 10 years of transfusion. There was significant negative correlation after 10 years of BT, which reflects that Hypocalcemia becomes more intense with prolonged transfusion therapy. The acquired levels of serum ALP did not portray any significant variations towards various durations or numbers of BT, which reflects that it may not be a significant marker in the aforementioned context as age-based outcome reflected no significant differences among ALP and calcium levels between younger patients of 5-10 years as compared to other patients of 11-15 years.

So, we prefer regular monitoring of levels of calcium among β-thalassemia patients, particularly as duration and frequency of blood transfusion increases, to reduce the intensity of hypocalcemia-associated complications.

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