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Comparative study of β-thalassemia major among the patients from urban and rural population in Hyderabad region

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Abstract

The thalassemia is a common etiological, microcytic and hypochromic anemia. It is an autosomal genetic disorder caused by mutations in α and β globin genes. In this experiment, hematological study conducted among the β-thalassemia major patients selected from urban (n=20) and rural (n=20) areas of Hyderabad. Patients from both regions were arranged into two age-groups (group I=2-7 years and group II= 5-11 years). Each group comprised on 10 males and 10 females (half rural and half city) in total 20 of confirmed β-thalassemia major patients. With hematological analysis showed significant variation, like as lowest red blood cells (RBCs) measured in male patients of both groups from urban and rural patients than all females. The hemoglobin (HB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCH), mean corpuscular hemoglobin concentration (MCHC) and platelets (PLT) showed variable ($p \geq 0.05$) abnormal values in all patients over normal reference values. The Hb also observed as reduced in all patients especially in 5-11 years aged male patients ($p \geq 0.05$). Meanwhile total iron contents and iron binding capacity (TIBC) noted reversed to Hb contents in all β-thalassemia major. In conclusion, rises in hematological parameters and iron overload causes to enhance antioxidant activity (AOA). The AOA may be beneficial for lowering the stringency of developed oxidative stress due to iron overload. It could be dependent on the type of AOA as well as their sources. Growth failure is common in thalassemia patients, while it is achievable with blood transfusion and optimal balanced nutritional status.

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Introduction

The thalassemia major (Cooley's anemia) is being an autosomal recessive blood disorder and prolongation in life of its patients depends on sever regular blood transfusion (Borgna-Pignatti *et al.*, 2004; Piomelli and Loew, 1991). This β-thalassemia is due to deficit (2 α-globin chains and 2 β-globin chains) or no biosynthesis of β-globin chains of hemoglobin (Hb) hetero-tetramer (Bank, 2005; Farashi *et al.*, 2015; Shishikura and Takami, 2001). These 4-protein chains of Hb are encoded 3 genes, 2 α-globin genes lined on chromosome 16 and 1 β-globin gene on chromosome 11. The α-thalassemia and β-thalassemia occur when their respective genes are defected (Jeffreys *et al.*, 1980; Wheeler *et al.*, 2004). Among major (genes from both parents) to minor thalassemia (gene from one parent), α-thalassemia major is most severe form. Its patients are able to born a child that could be normal at birth, while suffer with severe anemia in first year of life including some symptoms like as facial bone deformities, shortness of breath, fatigue, growth failure, yellowish skin appearance and shortness of breath. Patients with thalassemia minor (α or β) are asymptomatic but body produces abnormal Hb forms (Fucharoen and Winichagoon, 1992; Muncie and Campbell, 2009; Sripichai *et al.*, 2008).

The red blood cells (RBCs) with abnormal Hb are weaker oxygen carriers among thalassemia patients (Helley *et al.*, 1996). Life of these patients are constant dependent on blood transfusions. Continuous multiple transfusions have many side effects. Like as overloading of iron initiates secondary hemochromatosis in heart, liver, and endocrine glands, which leads to bone mineral acquisition deficits (Carmina *et al.*, 2004; Merchant *et al.*, 2011; Sanctis *et al.*, 2013). Growth retardation, low body mass index and impaired immune function has observed among thalassemia major children (Mohseni *et al.*, 2014; Rocher *et al.*, 2008; Vlychou *et al.*, 2016). This is result of interferences of various molecular mechanisms, which disrupt osteoblasts and osteoclasts balance and causes osteoporosis (high fracture risk) at adulthood stage (Blair and Carrington 2006; Nuntakarn *et al.*, 2009). Routinely addition of chelation treatment along the transfusion is best for prevention of toxic effects of overloading of iron which enhances thalassemia patient's survival rates (Baldini *et al.*, 2010; Casale *et al.*, 2014; Rosen and Klibanski, 2009).

Among the trace metals, iron is most causative agents and their accumulation causes oxidative damage in the blood cells especially erythrocytes (Eshghi *et al.*, 2007). It is because of unidirectional iron metabolism in human and its elimination via excretory route is limited. Therefore excess iron is deposited systematically among the vital body organs (Origa *et al.*, 2005; Rund and Rachmilewitz, 2005; Taher *et al.*, 2006). Proper estimation of trace elements remains valuable, as it might be involved in increasing the incidences of endocrinal abnormalities and adolescents from children to adult β-thalassemia patients. Influence of trace metals imbalance the body growth rate as well as imbalanced biosynthesis of hormones. Like as deficiency of zinc is a causative metallic agent of osteoporosis and endocrinosis (Mahyar *et al.*, 2010; Toumba *et al.*, 2007). By keeping in view, the aim of this study is to evaluate an intensive hematological parameters of samples of β-thalassemia major patients collected from Hyderabad city and nearby villages with control group.

Materials and methods

Sample source and selected thalassemia major patients

Twenty families of thalassemia major were referred for diagnosis to thalassemia unit. In total of 40, the 20 samples from village patients and 20 from city patients (10 males and 10 females from each region) from families of β-thalassemia major were selected for this study. These children were registered at Zanabia Blood Bank and Thalassemia Centre (ZBBTC), Hyderabad, Pakistan. The patients were categorized into two groups on the basis of their age. The group I includes the children with age from 2-7 years and group II with 7-11 years. All β-thalassemia major confirmed patients were followed with a regular schedule of blood transfusion.

Hematological screening of thalassemia

The fresh blood samples of selected patients were collected in anti-coagulated with EDTA-K_2 (ethylene diaminetetraaceticacid-dipotassium vials.

Blood samples were subjected for diagnosis of thalassemia. Hematological parameters of blood like as screening of disease counting CBC (complete blood count), Hb level, hematocrit (Hct), mean cell Hb (MCH), mean cell volume (MCV), MCH concentration (MCHC) and red blood cell distribution width estimated with an automatic hematology analyzer (Advia 2120, Bayer Diagnostics, Tarritown, USA) through automated hematology analyzer by following its manual (Harthoorn-Lasthuizen *et al.*, 1999; Mosca *et al.*, 2009; Verma *et al.*, 2014).

Estimation of hemoglobin

Hemoglobin was estimated with Drabkin's reagent by following HiCN procedure reported by Shah *et al.*, (2011) and Lewis *et al.*, (1991) methods. Shortly, dilute 20 µl blood in 4 ml Drabkin's reagent (or Cyanmethemoglobin**:** each of 200 mg potassium ferricyanide, 50 mg potassium cyanide, 140 mg potassium dihydrogen phosphate and 1 ml Ethyleneglycolmonopentylether as non-ionic detergent dissolved in distilled water one by one to make up to 1000 ml) at the rate of 1:200. Mixed thoroughly and stand at room temperature for 10 minutes. Absorbance was taken with spectrophotometer at 530. Drabkin's reagent was also used as blank, in preparation of standard solution of hemochromogens (12 G/dL) and also for dilution of test sample. The hemoglobin was calculated with this formula;

$$\textbf{Hb (g/dl)} = \frac{OD\ of\ test\ sample}{OD\ of\ standard\ solution}\ x\ Concentration\ of\ standard\ solution$$

Determination of iron

Iron contents in blood samples were determined by applying reaction of iron with ammonium thiocyanate (Eldin *et al.*, 2016) and OD was taken at double beamed UV-VIS Spectrophotometer (SP-3000 Plus model, Optima, Tokyo, Japan). Blood samples were subjected to wet digestion by mixing its 1.0 mL (=1.07 g) with 6.0 mL concentrated HNO_3 and 2.0 ml H_2O_2. The mixture was covered with watch glass and heated at 110°C for 30 minutes on hotplate. After that mixture was transferred volumetric flask and volume raised to 25 ml with deionized distilled water. The 0.5 ml of its filtrate was mixed with 2.5ml iron buffer reagent (220 mM Hydroxylamine Hydrochloride in Acetate Buffer, pH 4.5 with Surfactant).

After mixing first reading (A_1) was taken at 560nm. After that 0.05ml iron color reagent (3.6 mM Ferrozine in Hydroxylamine Hydrochloride) was added. Mixture solution was mixed and placed at 37°C in water-bath for 10 minutes than second reading (A_2) was recorded at 560nm. The blank and iron standard (40.0 g of NH_4SCN dissolved in 100 ml deionized distilled water) also processed same as above. The iron contents were calculated by applying below given formula;

$$Total\ iron\ (\mu g/dl) = \frac{OD\ A2\ of\ test\ sample - OD\ A1\ of\ test\ sample}{OD\ A2\ of\ standard\ sample - OD\ A1\ of\ standard\ sample}\ x\ Concentration\ of\ standard\ solution$$

Determination of TIBC (total iron binding capacity)

The TIBC was estimated in thalassemia patients (Al-Buhairan and Oluboyede, 2001; Yamanishi *et al.*, 2003). Sample reaction mixture was prepared by pipetting 2.2 ml Tris- buffer (500 nmol/l), 0.3 ml standard (500 µg/dl ferric iron), 0.3 ml sample. For blank iron free water and standard is same to sample reaction mixture without sample. Stand the reaction mixtures at room temperature for 1 minute than measure absorbance (A_1) at 560 nm. Exact 100 µL iron color reagent was added and mixed well. Reaction mixture was incubated at 37°C for 10 minutes. The second OD (A_2) was read at 560 nm.

$$a.\ Excess\ iron\ (\mu g/dl) = \frac{OD\ A2\ of\ test\ sample - OD\ A1\ of\ test\ sample}{OD\ A2\ of\ standard\ sample - OD\ A1\ of\ standard\ sample}\ x\ Concentration\ of\ standard\ sol$$

b. UIBC (µg/dL) = 500 (the total iron added in µg/dL) - ExcessIron (µg/dL)

c. TIBC (µg/dL) = Serum Iron (µg/dL) + UIBC (µg/dL)

Determination of antioxidant activity (TAA)

Total antioxidant (TAA) was measured in fresh blood sample (Evenson and Carmack, 1979; Korotkova *et al.*, 2013; Sun *et al.*, 1988). First it was centrifuged at 2,000 rpm for 10 minutes, than its 0.2 ml was mixed in 2 mL Tween-80 (1%) and incubated at 40° C for 48 hours. 0.4 ml NaCl (0.9%l) was added and centrifuged at 3000 rpm for 10 mines (repeat twice). Supernatant was discarded and 0.9 ml dH_2O was added and mixed to ready the hemolysate.

Each of 2 ml Tween-80, 0.2 ml ferrous sulfate solution (1 mM $Fe_2(SO_4)_3$, 0.2 ml ascorbic acid (10 mM ascorbic acid) 0,1 ml hemolysate (or 0.2 ml plasma) were poured in dark glass vial and mixed thoroughly. Same were control and standard but in place of sample, distilled water and uric acid (1 mmol uric acid in 5 mmol NaOH) were replaced. Reaction mixture was incubated for 48 hours at 40°C. Its 2 ml was mixed with 1 ml 20% TCA (trichloroacetic acid in dH_2O) than it was centrifuged at 8000 rpm for 15 min. the 1 ml of supernatant was mixed in 2 ml TBA (0.8% Thiobarbituric acid in 50 mmol NaOH) and boiled for 15 min. After cooling to room temperature, OD of upper phase was taken at 532nm against dH_2O.

Statistical Analysis

Data of this study was analyzed CoStat (version 3.03) *CoHort* software, Berkeley, USA. Significant means differences among the normal to thalassemia patients were subjected for further assessment by Duncan Multiple Range (DMR) test at 5% (Behrens, 1997; Henley, 1983; Quinn and Keough, 2002). For descriptive statistics like as means of estimated data from biochemical analysis and standard deviation are calculated for the purpose as they used to describe demographic characteristics of (urban and rural patients) β-thalassemia major patients of different age groups. The data of each hematological parameter was expressed in mean as the means and standard deviations (SD) of data are found by Benetou *et al.*, (2006).

Results and discussion

The β-thalassemia major patients has been reported in Africa (0.9%), Cyprus (14%), Italians (5%), Northern Europe (0.1%), Portuguese (3.5%), Sardinia (12%), South East Asia (39%) including Pakistan (5.4%) (Cao and Galanello, 2010; Flint *et al.*, 1998; Vichinsky, 2005; Weatherall, 2011). Its 81.7% patients are being outcome of consanguineous marriages (Baig *et al.*, 2008; Ishaq *et al.*, 2012) and due to unawareness their pregnancy (carrier couple) is available at risk to born 25% affected child (Baig *et al.*, 2006). Blood transfusion system has been adopted for just survival of patients (Nosheen *et al.*, 2015) but it is also controlled successfully with bone marrow transplantation (Lucarelli *et al.*, 1999; La Nasa *et al.*, 2005).

Due to unavailability as well as unaffordable bone marrow transplantation facility for β-thalassemia disorder preventions. Mostly patients go to death, while premarital and proper counseling with thalassemia carriers may be beneficial (Nosheen *et al.*, 2015). In this experiment, certain hematological parameters are studied among the local male and female β-thalassemia major patients with 2-11 years aged of Hyderabad region. Whenever a patient got same blood hematology as suggested in present study should get worried about himself as well as for his or her transcends.

Of the 40 samples of β-thalassemia major patients [20 males (♂) and 20 females (♀)] from urban (Hyderabad) and rural (villages around Hyderabad) areas were arranged into two age groups. The group I with age of 2 to 7 years (5 ♂ and 5 ♀ village; 5 ♂ and 5 ♀ city) and group II with 5 to 11 years (5 ♂ and 5 ♀ village; 5 ♂ and 5 ♀ city) were diagnosed and subjected for comparative study. The β-thalassemia major is observed quite variable in rural to city regions. This variation is due to its recessive inheritance, which is highly prevalent in families preferred for consanguineous marriage. Among these collected patients, severe anemia was observed which results due to low hemoglobin concentration than normal reference values. It was lowest in village or rural males of both age groups of 2-7 years and 5-11 years showed 05.998±0.194 g/dl and 05.42±0.312 g/dl respectively than city males of same ages (07.02±0.357 g/dl and 06.72±0.401 g/dl). Very similar trend of hemoglobin was estimated in females of rural and urban β-thalassemia major patients (Table 1). In according to gender and age group based comparisons, hemoglobin is higher ($p \geq 0.05$) in females of city and village patients (Table 1, Fig 1).

According to the hematological parameters, urban β-thalassemia major patients revealed significant variation within and among groups to the rural patients, while both are exceeding over to the reference values. This difference could be due to variation in nutrient imbalanced conditions of available diet to patients from urban and rural areas.

Table 1. Analysis of hematological parameters among β-thalassemia major patients from urban (n=20) and rural areas (n=20) of Hyderabad region (village and city) of different gender (10 males and 10 females) and two age groups (group I= 2-7 years and group II= 5-11 years).

Parameters *(Rf. Values)*	♂/♀	Village Patients		City Patients		*F-sig.*
		2-7 years	*5-11 years*	*2-7 years*	*5-11 years*	
WBCs (10^9/L)	♀	^{a}10.62±0.364	^{cd}06.70±0.416	^{d}05.91±0.401	^{d}07.52±0.542	30.93^{***}
(3.80-11.20)	♂	^{bc}07.30±0.488	^{b}06.13±0.261	^{a}9.942±0.358	^{d}06.38±0.218	
LY (%)	♀	^{c}36.86±1.301	^{b}43.44±2.298	^{a}56.54±3.040	^{b}35.40±01.40	23.29^{***}
(20.27-55.48)	♂	^{a}55.08±1.844	^{c}45.45±1.952	^{b}47.70±3.864	^{c}33.86±1.578	
MO (%)	♀	^{d}2.640±0.260	^{cd}03.28±0.515	^{a}06.04±0.702	^{bc}02.82±0.338	13.01^{***}
(4.40-12.13)	♂	^{b}04.06±0.240	^{cd}03.76±0.229	^{b}04.20±0.383	^{bcd}03.52±0.132	
GR (%)	♀	^{b}59.68±0.675	^{cd}53.12±0.875	^{e}36.62±0.856	^{c}59.18±3.373	37.20^{***}
(35.00-74.43)	♂	^{e}41.16±1.714	^{b}54.80±2.429	^{d}49.90±3.825	^{a}65.02±1.964	
RBCs (10^{12}/L)	♀	^{ab}3.414±0.136	^{bc}03.23±0.191	^{a}03.71±0.225	^{e}02.19±0.190	11.73^{***}
(3.46-5.07)	♂	^{de}02.63±0.181	^{de}02.38±0.101	^{bc}3.138±0.103	^{cd}02.82±0.194	
Hb (g/dl)	♀	^{a}8.260±0.279	^{cd}06.58±0.275	^{bc}07.16±0.452	^{e}07.36±0.181	12.33^{***}
(9.20-13.20)	♂	^{de}5.998±0.194	^{b}05.42±0.312	^{bc}07.02±0.357	^{bcd}06.72±0.401	
HCT (%)	♀	^{a}24.90±1.108	^{b}22.44±1.014	^{ab}23.32±0.858	^{ab}22.04±1.284	10.27^{***}
(30.10-43.00)	♂	^{c}18.44±0.612	^{b}23.92±0.798	^{b}22.08±0.910	^{c}18.42±0.920	
MCV (fl)	♀	^{a}60.96±1.765	^{cd}53.43±1.584	^{a}60.032±2.236	^{cd}53.19±1.413	8.197^{***}
(66.06-95.60)	♂	^{bc}55.90±0.751	^{bc}56.47±1.539	^{ab}21.04±0.477	^{d}52.23±0.868	
MCH (pg)	♀	^{bc}21.04±0.477	^{b}22.24±1.014	^{bc}19.62±0.723	^{bc}21.84±0.318	3.944^{**}
(21.10-31.23)	♂	^{c}19.84±0.606	^{bc}22.04±0.494	^{a}19.62±0.723	^{bc}21.44±0.463	
MCHC (g/dl)	♀	^{a}35.22±1.747	^{a}34.88±0.723	^{a}36.74±0.786	^{a}35.68±0.872	0.616^{ns}
(28.70-34.60)	♂	^{a}34.86±0.823	^{a}35.30±0.758	^{a}35.96±0.862	^{a}35.20±0.321	
PLT (10^8/µl)	♀	^{b}128.8±4.309	b137.0±3.764	^{b}133.0±3.002	b123.6±5.896	9.408^{***}
(160- 454)	♂	b123.0±5.066	^{b}135.4±4.539	^{a}125.8±5.707	^{b}116.2±2.898	

♀: Female; ♂: Male; WBCs: White blood cells; LY: Lymphocytes; MO: Monocytes; GR: Granulocytes; RBCs: Red blood cells; Hb: Hemoglobin; HCT: Hematocrit; MCV: Mean corpuscular volume; MCH: Mean corpuscular hemoglobin; MCHC: Mean corpuscular hemoglobin concentration; PLT: Platelets; Ref: Reference; sig: Significance.

It could be dependent on the value of income of patient's family. Higher rate of hemoglobin in patients from urban area may be result of usage of nutritionally balanced diet. The thalassemia patients showed significantly lower values than reference values of hemoglobin (Hb), hematocrit (HCT), mean corpuscular hemoglobin (MCV) and mean corpuscular hemoglobin concentration (MCHC), while white blood cells (WBCs), lymphocytes (LY) and platelets (PLT) were higher (Table 1) (Adel *et al.*, 2015; Karim *et al.*, 2016). Among the β-thalassemia major patients, iron over load has observed among all patients ($p \geq 0.05$), which causes oxidative stress. Higher values of TIBC among the males and females from village as well as city aged based groups was higher ($p \geq 0.05$) than normal values especially in male and female from village (Fig 1). This increasing trend of iron causes destruction of RBCs. The highest antioxidant activity (AOA) was found in city females followed by city males and it was lowest ($p \geq 0.05$) among both females and males patients from village (Fig 2).

Function of antioxidants is lower the ratios of free radicals. It means that if AOA is lower than normal values in thalassemia patients, iron accumulation is rising. It results into overloading of iron, which leads to increase the oxidative stress and death of RBCs ultimately.

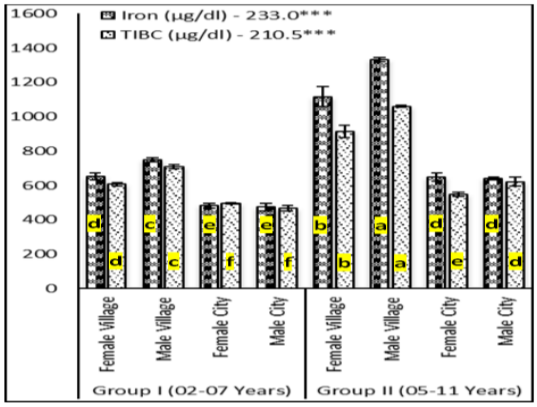

Fig. 1. Iron contents (µg/dl) and total iron binding capacity (TIBC, µg/dl) in blood samples of the β-thalassemia major patients from urban (n=20) and rural areas (n=20) of Hyderabad region (village and city) of different gender (10 males and 10 females) and two age groups (group I= 2-7 years and group II= 5-11 years).

The TIBC values of village males were much higher than normal and increased than urban patients. This was evident of increase release of ferritin with increase RBCs destruction. Antioxidants inhibit free radicals and are found in lower amount in thalassemia patients than normal. The antioxidant activity of urban patients (females and males) is in increasing order from village males to city females and frequency is lowest in rural (males) patients. The oxidative stress is increased due to free radicals production by secondary iron overload. Present study results showed some interesting findings that male thalassemia parents give more medical attention than females because male birth is prime importance in Pakistani culture and other reason is that male is also considered as head of family (Javed, 2013). The total iron-binding capacity (TIBC) is to measure the concentration of transferrin of serum because the free iron binds with some proteins. Low TIBC causes inflammatory disorders (Feldman *et al.*, 1981; Ottenjann *et al.*, 2006; Smith and Cipriano, 1987; White *et al.*, 2012), while its increased level indicates due to excess iron overload (Kasvosve and Delanghe, 2002; Moosavian *et al.*, 2010; Roberts *et al.*, 1999) if patients with chronic hepatopathy (Jacobs *et al.*, 2000).

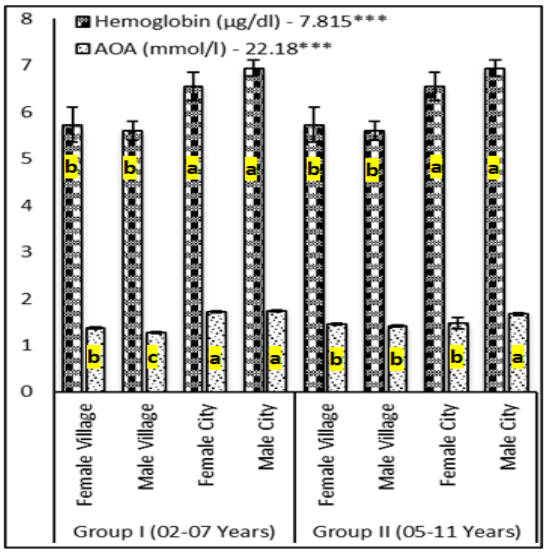

Fig. 2. Concentrations of hemoglobin (µg/dl) and antioxidant activity (mmol/l) among the β-thalassemia major patients from urban (n=20) and rural areas (n=20) of Hyderabad region (village and city) of different gender (10 males and 10 females) and two age groups (group I= 2-7 years and group II= 5-11 years).

The β-thalassemia major patients have a basic defect of reduction or no production of β-globin chains, which leads to excessiveness of α-chains relatively. An imbalanced combination of excess α-chains with residual β-chains in β-thalassemia patients leads to develop oxidative stresses, which causes proteolysis. Production of antioxidants could be function for the prevention of proteolysis by accommodating the free cations including iron. This mechanism is workable at certain limit, ultimately both oxidation and proteolysis causes to inhibit as well as death of precursors RBCs in bone marrow. It is hallmark of β-thalassemia major due to infectivity of erythropoiesis. The released RBCs in peripheral blood undergo to hemolysis by reticuloendothelial system as being further contribution to anemia (Verma *et al.*, 2014). Such increasing β-thalassemia major patient's burden is higher, while available treatment option till date are blood transfusion and stem cell transplantation. First ongoing practice but latter is availed by very few patients only who can bear in foreigner's hospitals. Rest are waiting for mercy of god for death or help to treat. Meanwhile, repeated blood transfusions could be their survivor but totally subject to an available voluntary for a variety of other complications related with blood transfusion unconsciously. Like as allergies, febrile non haemolytic transfusion reaction, haemolytic transfusion reactions, alloimmunization, lung injury, transmission of infections like HCV, HIV, HBsAg and graft versus host disease. Further, iron overload is main and initial adverse event among patients depending on repeated blood transfusion (Hoffbrand *et al.*, 2012).

Conclusions

The β-thalassemia major and its sub-types are identifiable with the estimation of hematological parameters. In this study, all selected patients are affected with β-thalassemia major. Severely affected hematological values (HB, HCT, MCV, MCHC, PLT etc) observed in rural patients than urban. Variable values of various bio-contents of thalassemia patients, the hemoglobin, iron, TIBC and antioxidant activity in rural (lower values) and urban β-thalassemia patients (higher) is the result ($p \geq 0.05$) of different quantity and quality of nutrient intake.

It is also including the unavailability of health facilities, lack of knowledge about thalassemia, improper iron chelation and no control programs for disease in rural areas especially. Prenatal screening either thalassemia diseased or carrier and their sub-sequent offsprings can be a best way to reduce the ongoing frequency of thalassemia. Just by discouraging the cousin marriages. Monitoring program and special designed treatment including quality food which induces RBCs development and free iron engulfing agent from blood will be useful in providing optimal care.

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Conflict of interest

No conflict of interest.

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