

Role of Vitamin C Supplementation on Iron Overload and Oxidative Stress in Beta Thalassemia Major Patients in Maysan Province-Iraq

Jafar Naser Hussien¹, Sami Rahem Al-Katib² & Salah Hashim Al-Zuhairy¹ Department of Pediatrics, College of Medicine, Maysan University Department of Physiology and Medical physics, College of Medicine, Kufa University e-mail: jafarbahadili@yahoo.com

Keywords: Vitamin C, Thalassemia major, Iron overload, Oxidative stress

ABSTRACT

Background: Vitamin C is a well-known powerful water soluble antioxidant and very powerful iron absorption enhancer. It was used for a long period in the management of thalassemia major (TM) patients hoping to decrease the oxidative stress initiated by iron overload resulted from hemolysis of erythrocytes and frequent blood transfusions (BT). Iron overload from BTs may be exacerbated in patients due to increased absorption of iron from the diet in response to ineffective erythropoiesis or vitamin C supplementation.

Aim: To evaluate the impact of vitamin C supplementation on the iron overload and oxidative stress in beta thalassemia major patients

Methods: Fifty five children were investigated in this study. Thirty two of them were patients with TM on frequent BTs and oral chelating drug (deferasirox), they were diagnosed by hemoglobin electrophoresis with a mean age of 9 ± 4.3 y. Patients were attendees of the Blood Disease and Thalassemia center in Maysan Province-Iraq in the period between November 2013 and

November 2014. Twenty three apparently healthy children with a mean age of 8 ± 3.2 y were included as a control group. Patients were subjected to vitamin C supplementation (200mg/day) for 2 months. Serum; vitamin C, iron, malondialdehyde (MDA), ferritin and UIBC and complete blood count were determined.

Results: There were significant increases of serum iron ($P<0.05$) and ferritin ($P<0.05$) levels with insignificant increase of serum MDA levels in samples of patients before supplementation when compared with those of the control groups. However, a significant decrease in serum vitamin C levels ($P<0.05$) and serum UIBC values ($P<0.05$) were evident during a similar comparison. The results of second samples, in comparison with the results of first samples referred to a statistically significant increase in serum vitamin C and serum iron ($P<0.05$), non-significant increase in serum ferritin and MDA and non-significant decrease in UIBC. The complete blood count remained the same in both samples.

Conclusion Vitamin C is a powerful enhancer of iron absorption from GIT and releaser from

intracellular stores than antioxidant agent. Vitamin C must be given with caution and continuous supervision to TM patients.

Recommendation: It is essential to study both smaller and larger doses than 200mg with a longer periods and expanded age groups.

Introduction

Thalassemia is a well-known hereditary disorder affecting the production of normal hemoglobin. The more severe form of the disease is TM, in which there is a complete absence of beta chain due to gene deletion and excess production of alpha chain and accumulation of Heinz bodies in the erythrocyte (RBC). TM patients almost need frequent BTs for their survival. Hemolysis of RBC due to defective hematopoiesis and frequent BTs result in iron overload due to the lack of effective excretion pathway for the excess iron [1]. Iron overload presented by increased serum iron and serum ferritin levels and decreased UIBC levels yields an oxidative stress through production of many reactive oxygen species (ROS) specially the hydroxyl radical and this lead to exhaustion of the intracellular antioxidant defense mechanism and protein, DNA and lipid degradation. Protein and DNA degradation produces many intermediate and end products which are non-specific and easily changed by the normal metabolic processes [2].

Lipid peroxidation which affects mainly the membranes occurs later in the process and give us an impression about the antioxidant status and tissue damage due to the oxidative stress. Lipid peroxidation process, especially

of the polyunsaturated fatty acids (PUFAs) which is present mainly in the cytoplasmic and intracellular membranes, produces so many hydroperoxide compounds like MDA, propanal, hexanal, and 4-hydroxynonenal (4-HNE). MDA gives the most appreciation of the lipid peroxidation process for it is more stable than other hydroperoxides [3].

Vitamin C is a potent water soluble antioxidant. It is used as a supplement to withstand the oxidative stress in TM patients. Vitamin C is proved to be a potent enhancer for iron absorption from gastrointestinal tract GIT via the reducing power of ascorbate and preventing formation of insoluble and un-absorbable iron compounds in the GIT [4]. Non-heme iron is the target of vitamin C absorptive enhancement [5]. The facilitating effect of vitamin C is a dose related. In one study, in which increasing amounts of vitamin C ranging from 25 to 1000 mg were added to a liquid formula meal containing 4.1mg non-heme iron, iron absorption increased progressively from 0.8% to 7.1% respectively [6]. Due to the enhancement of non-heme iron absorption by vitamin C, a theoretical concern is that high vitamin C intakes might cause excess iron absorption. In healthy individuals, this does not appear true [7]. However, in individuals with hereditary hemochromatosis and TM, chronic

consumption of high doses of vitamin C could exacerbate iron overload and result in tissue damage [8].

The oxidative damage presented by MDA measurement in TM patients receiving vitamin C remains as a matter of debate. Some researchers concluded that small doses of vitamin C have no harm to TM patients, while others restrict its supplementation with chelating drugs only [9]. Vitamin C is routinely given to TM patient to overcome the oxidative stress initiated by the iron overload produced by frequent BT and defective hematopoiesis. It also enhances the release of iron from its stores to chelating agents. Several studies on the role of vitamin C in increasing the absorption of iron from GIT suggested the harmfulness of this role in increasing the iron overload and hence the deterioration of the oxidative stress on tissues. In the current study, the impact of vitamin C supplementation on the iron overload and oxidative stress was evaluated in thalassemia major patients.

Methods

Thirty two patients with TM who were diagnosed by hemoglobin electrophoresis and positive family history, and on regular BT and oral chelating agents (deferasirox) were studied. The mean age was 9 ± 4.3 y. They

were attending the Center of Blood Diseases and Thalassemia in Maysan Province for BT, investigations, consultation, and regulation of their therapy. Patients had no cardiac problems, no obvious endocrinological problems, nonsmokers, non-sickling, and had no splenectomy. Patients with diabetes mellitus and hypothyroidism were excluded by after measurement of the fasting blood glucose and T3, T4, and TSH levels. A history of the patient's drug taking, complications had been arisen through the periods of treatments and exclusion of infective hepatitis are put in our consideration. After getting agreements from the patient's parents, and made sure that patients didn't receive any vitamin C supplementations, apart from normal dietary sources for previous 6 months, vitamin C was given in a dose of 200mg/day in two divided doses for two months starting immediately after taking the blood. Twenty three healthy children were selected randomly to be a control group, their mean age was 8 ± 3.2 y. No vitamin C was supplemented for the control group.

Blood samples are taken from the patients through the IV line which is connected to them for the blood transfusion, 1.5 ml are collected in tubes containing EDTA as anticoagulant to be used for the determination

of complete blood count (RBC, Hb, HCT, MCV, MCH, MCHC, RDW%, WBC, Neutrophil%, Lymphocyte%, Monocyte%, Eosinophil%, Basophil % and Platelet). Another sample of blood (2-3.5 ml) was collected in a plane gel tube and centrifuged at 3000 xg for 10 min and the serum was collected and distributed as follow: 250µml in small plastic tubes for determination of MDA, 250µml in small plastic tubes for determination of serum iron and serum UIBC, 250µml in small plastic tubes for determination of serum ferritin and 0.5ml in plane tubes containing 2ml freshly prepared metaposphoric acid (MPA) for determination of serum vitamin C.

Plasma vitamin C levels were estimated calorimetrically at Maysan College of Medicine Laboratories using a DTCS reagent prepared by mixing copper sulfate, thiourea, and dinitrophenyl hydrazine, DNPH and copper in a 1:1:20 ratio (Five milliliters of 5% thiourea and 5ml of 0.6% copper sulphate were mixed with 100 ml of DNPH solution) as described by Teitz [10]. The reference values of vitamin C is 0.6–2 mg/dL with optimal value of 0.9 mg/dL [11].

Serum iron level was determined in Al-Sadr teaching hospital laboratories, by using ARCHITECT c4000 Clinical Chemistry Analyzer (ABBOTT COMPANY). The

reference values of serum iron is 50–120 µg/dL [12].

For the determination of ferritin, serum sample was diluted initially by special reagent (provided with the kit) in 1:9 proportion because high values of serum ferritin level in the blood samples of TM patients could not be detected colorimetrically (Upper detection limit in the kit is 1200 ng/ml). We diluted 50 µml of serum in 450 µml of the reagent. It was measured by MINI-VIDUS ELISA method. Results obtained were multiplied by 10. The upper limit value is 12000 ng/ml. The reference values for serum ferritin in healthy subjects is 12-300ng/mL (27–670 pmol/L) in males and 12-150 ng/mL (27–330pmol/L) in female [13].

For measurement of UIBC, serum was added to an alkaline buffer/reductant solution containing a known concentration of iron to saturate the available binding sites on transferrin. The iron that remained free after transferrin saturation was reduced to a ferrous state and let to react with Ferene-S to form a stable complex and the color intensity is measured at 580-600nm. The device used for measurement of UIBC is ARCHITECT c4000 Clinical Chemistry Analyzer (ABBOTT COMPANY). The solutions and compounds used were also provided by the same company. After adding 4 tablets of the

compound to the solution, and incubating it for two hours we added 4ml of UIBC solution and measured it by the device. UIBC is therefore determined by subtracting the quantity of unbound iron from the total added quantity.

The reference values of UIBC in normal individuals is 160-310 $\mu\text{g/dL}$ [12].

MDA level in serum was measured by the employment of the competitive inhibition enzyme immunoassay technique (CUSABIO Company). The assay depends on antibody specific for MDA, pre-coated on a microplate. Standards were prepared. The standards and samples were pipetted into the wells with a Horseradish Peroxidase (HRP) conjugated MDA. A competitive inhibition reaction was launched between MDA (Standards or samples) and HRP-conjugated MDA with the pre-coated antibody specific for MDA. The more amount of MDA in samples, the less antibody bound by HRP-conjugated MDA. Following a wash to remove any unbound reagent, a substrate solution was added to the wells and color developed in opposite to the amount of MDA in the sample. The color development was stopped and the intensity of the color was measured colorimetrically by wave length 450nm. The reference values is around 7.5

$\mu\text{g/ml}$. The control group values in our study were regarded as the normal.

The analysis of data was carried out using the available Statistical packages for social science, version 16.0(SPSS-16.0). Independent t-test was used for testing the significance of association between variables under study. Statistical significance was considered whenever the p-value was equal or less than 0.05.

Results

The serum level of vitamin C determined before the supplementation of vitamin C revealed highly a significant depletion ($P<0.001$) in comparing with the reference values (0.6-2mg/dL) and optimal normal value of serum vitamin C (0.9 mg/dL) (Mayo Clinic, 2012), in spite of normal diet which contains the proper amount of vitamin C that prevents children from vitamin C deficiency. Some patients showed very low values beyond the normal values and others showed near zero level. The average value of vitamin C in this study was 0.22 mg/dL with a standard deviation of 0.15 (Table 1). After two months of vitamin C supplementation with 200 mg/day in two divided doses the average level reaches only the lower normal value with a mean of 0.44 mg/dL and a standard deviation of 0.16 (Table 1). The

effect of vitamin C supplementation on the level of vitamin C is shown in Table2. Four patients (12.5%) showed slight decrease in serum vitamin C values.

Table 1

Mean serum ascorbic acid level before and after vitamin C supplementation as compared with levels in healthy individuals

	Ascorbic Acid Mean \pm SD (mg/dL)	Optimal level (mg/dL)	Increment
Before supplement	0.22 \pm 0.15*	0.9	300%
After supplement	0.44 \pm 0.16*	0.9	104%

* p value > 0.001

Table 2

Mean serum ascorbic acid values in pre and post treatment samples

	N	Serum vitamin C Mean \pm SD	P value
Before supplementation	32	0.22 \pm 0.15	< 0.01
After supplementation (20mg/day)	32	0.44 \pm 0.16	

The mean serum MDA value initially obtained from patients before vitamin C supplementation exhibited non-significant increase in comparison with the control group values (Table 3). In comparison between serum MDA levels after vitamin C supplementation with control group values, serum MDA showed non-significant increase (Table 3). Results of the effect of vitamin C supplementation (200mg/day) for two months on serum MDA levels in TM patients are illustrated in Table 3. Anyhow, there was fluctuation in the raised values of MDA

between patients as presented by the standard deviation values before and after supplementation, which may be due to several factors such as severity of the disease, hidden infection, ingestion of antioxidants, improper doses of vitamin C and errors in handling or processing of samples during assessment, but the overall results indicate a non-significant increase in MDA serum values. Eight patients out of 32 patients (25%) showed a decline in serum MDA level.

Serum ferritin levels showed significant increase in comparison with those of the control group. The mean level of serum

ferritin before vitamin C supplementation is 3370 ng/ml with a standard deviation of 2454 ng/ml. The mean value was about 49 folds than the control group (Table 4). After two months of supplementation with vitamin C (200mg/day in two divided doses), the mean value was about 52 fold than the control

group (Table 4). The mean value of serum ferritin elevated to 3618 ng/ml with a standard deviation of 1756 ng/ml (Table 4). Only two patients out of 32 patients (6.2%), serum ferritin level demonstrated a decline after vitamin C supplementation.

Table 3

Serum malondialdehyde levels in thalassemia major patients before and after supplementation with vitamin C (200 mg/day) and in the control group

	N	Serum MDA Mean \pm SD (μ g/ml)	SE	P value
Before supplementation	32	10.3 \pm 8.3	1.4	0.40
Control	23	8.7 \pm 4.8	1.0	
After supplementation	32	12.2 \pm 7.6	1.3	0.054
Control	23	8.7 \pm 4.8	1.0	
Before supplementation	32	10.3 \pm 8.3	1.4	0.33
After supplementation	32	12.2 \pm 7.6	1.3	

SE= standard error

The serum iron level showed an increase in its level comparing with the control group in about one fold ($P < 0.01$). The mean value obtained before vitamin C supplementation is 178 μ g/dL with a standard deviation of about 68 μ g/dL (Table 5). After supplementation with vitamin C in a dose of 200 mg/day for two months, the serum iron level increased

significantly ($P > 0.007$) to a mean of 229 μ g/dL with a standard deviation of about 78 μ g/dL. The percentage of the increment is about 29% compared with pre-treatment result (Table 5). In the control group, the mean value and the standard deviation are about 85, 23 respectively.

A statistically significant ($P < 0.001$) decrease in the levels of serum UIBC of TM

patients (in both pre and post vitamin C supplementation samples) were observed when compared with the control group. After two months of vitamin C supplementation the mean value of serum UIBC decreased non-significantly in comparison with the first sample taken before supplementation. In one patient, unacceptable value had been obtained and discarded from the statistics. In 14 out of the 31 patients, values were below the measurable limit of the device ($41 \mu\text{g/dL}$) in the pre-treatment samples. In 16 out of the 31 patients, values were below the measurable limit of the device in the post-treatment samples (Table 6). In 14 out of the 31 patients, values were below the measurable limit of the device ($41\mu\text{g/dL}$) in the pre-treatment samples. In 16 out of the 31 patients, values were below the measurable limit of the device in the post-treatment samples.

The initial samples (before vitamin C supplementation) results of complete blood count (CBC) indicated a mild decrease in the number of RBC (3.21 ± 0.52) $\times 10^6$ cell/ μL compared with the reference values (3.6-4.5) $\times 10^6$ cell/ μL . The Hb values were markedly decreased. The mean corpuscular volume (MCV) and mean corpuscular Hb which are considered as pointers in the diagnosis of TM showed a mild decline with a mean and SD of

74.58 ± 3.91 and 26.5 ± 1.99 respectively (Table 7).

Discussion

Results of serum ascorbic acid levels before vitamin C supplementation obtained in this study is agreed with findings of most studies over the world and support the idea of multi-nutrients deficiencies in beta thalassemic patients previously stated by FAO and WHO [13,17]. The decline of vitamin C levels observed may be attributed to so many factors. Kent et al., have stated that ascorbic acid acts mainly by reducing the capacity and mobilize excess iron from tissue stores [18], also acts as an antioxidant toward lipids in iron-overloaded human plasma in vitro [19]. The cause of the decline may be an increase consumption of the antioxidants (vitamin C) to overcome the oxidative stress of the iron overload in thalassemic patients or it may be due to the increased demand to vitamin C in the hyper-metabolic status of patients. Altering the absorptive power of epithelial cells and endothelial cells to vitamin C may be an additional factor of the deficiency.

Table 4:

Serum ferritin levels before and after supplementation with vitamin C (200mg/day)

	N	Serum ferritin Mean \pm SD (ng/ml)	SE	P value
Before supplementation	32	3396.9 \pm 2454.1	433.8	0.001
Control	23	69.1 \pm 31.9	6.6	
After supplementation	32	3617.9 \pm 1755.6	310.3	0.001
Control	23	69.1 \pm 31.9	6.6	
Before supplementation	32	3396.9 \pm 2454.1	433.8	0.68
After supplementation	32	3617.91 \pm 1755.6	310.3	

SE= standard error

Table 5:

Serum iron levels before and after supplementation with vitamin C (200mg/day)

	N	Serum iron Mean \pm SD (μ g/dL)	SE	P value
Before supplementation	32	178.4 \pm 67.6	11.2	0.001
Control	23	85.1 \pm 22.7	4.7	
After supplementation Control	32	228.8 \pm 77.6	13.72	0.001
	23	85.1 \pm 22.7	4.74	
Before supplementation	32	178.4 \pm 67.6	11.95	0.007
After supplementation	32	228.8 \pm 77.6	13.72	

SE= standard error

It is evident that serum ascorbate level didn't reach the reference value (0.6-2mg/dL) [11], despite the supplementation with vitamin C for two months. This finding indicated that the body store of vitamin C was quite depleted to the point that such dose hardly met the actual need of tissues and vital metabolic processes needed by thalassemic

patients. This finding is corresponding with the conclusions of Dissayabutra who found that after three months of vitamin C intake, plasma vitamin C was significantly increased [20].

Previous studies demonstrated significant rises in serum iron and ferritin levels in TM patients. Such elevation was interpreted as a defective hematopoiesis with elaboration of large amount of iron in the body as well as

the frequent blood transfusions needed for survival of patients in the absence of proper pathway for iron excretion in the body. Although serum ferritin is not as reliable as liver iron concentration (LIC) for estimating total body iron stores, as serum ferritin levels are affected by common processes, such as : Infection, inflammation, vitamin C deficiency and oxidative stress, it is till now stay the acceptable method for estimation of iron store level in most countries [21].

The current results of serum iron and ferritin levels and the UIBC were consistent with results of Sharma who used 500 mg of vitamin C twice daily after meals to increase hemoglobin and serum ferritin in Indian vegetarians and concluded that vitamin C was more effective at increasing iron status than iron supplements alone [22]. These rises are explained by the presence of various doses of phytate in the normal food that reduce iron absorption by 10 to 50%. Vitamin C counteract the effect of phytate on iron absorption. Also the results of this study was agreed with those obtained by Siegenberg who found that 50 mg of vitamin C counteracted the phytate, and 150 mg of vitamin C increased iron absorption to almost 30%, and in the presence of a large dose of tannic acid in the food, 100 mg of vitamin C increased iron absorption from 2 to 8% [23].

Results also are consistent with those of Seshadri who gave 2 doses of 100 mg of vitamin C/day with the food for 60 days, and obtained a drastic improvement of anemia, with a full recovery in most of patients [24]. UIBC values in TM patient is very low due to the free iron found intracellularly and extracellularly and bind to unoccupied sites [2]. After two months of vitamin C supplementation, the mean value of UIBC decreased due to the marked increase in iron level.

Results of serum MDA levels exhibited non-significant rise in patients with respect to the control group. These results are disagreed with those obtained by Das who found a significant increase in the level of serum MDA in patients with TM as compared to the control group [25]. However, results were also coincide with those obtained by Cighetti who concluded that the peroxidative status generated by iron overload in thalassemia patients highlight the rapid formation of marked amounts of free MDA despite the chelation therapy. In the TM patients, the free MDA levels correlated positively with serum ferritin, whereas the total MDA concentration correlated positively with non-transferrin binding iron NTBI [26].

Table 6:

Serum unsaturated iron binding capacity levels (UIBC) before and after supplementation of vitamin C (200mg/dL)

	N	UIBC Mean \pm SD (μ g/dL)	SE	P value
Before supplementation	32	100.4 \pm 97.0	17.1	0.001
Control	23	236.1 \pm 49.6	10.3	
After supplementation	31	79.6 \pm 65.1	11.5	0.001
control	23	236.1 \pm 49.6	10.3	
Before supplementation	32	100.4 \pm 97.0	17.1 11.5	0.3
After supplementation	31	79.6 \pm 65.1		

SE= standard error

Supplementation of vitamin C leads to increase its absorption and mobilization, resulting in enhanced oxidative stress and peroxidation of lipid by Fenton reaction [27]. On the contrary, Dissayabutra have observed no effect on MDA levels after three months of supplementation with vitaminC, vitamin E and glutathione to TM patients [20]. Han-Yao Huang stated that, supplementation with vitamin C reduced lipid peroxidation in normal volunteers [28]. Kashinakunti mentioned a rise of MDA level in smokers with acute myocardial infarction after vitamin C supplementation due to gene–environmental factors [29].

In conclusion, vitamin C is a powerful enhancer of iron absorption from GIT and releaser from intracellular stores than antioxidant agent. Vitamin C must be given with caution and continuous supervision to TM patients. It is essential to study both smaller and larger doses than 200mg with a longer periods and expanded age groups.

Table 7:

The mean complete blood count values in patients before and after supplementation with vitamin C (200mg/dL)

	Before supplementation	After supplementation	Reference value
RBC (cell/ μ L)	3.21 \pm 0.52	3.46 \pm 0.66	3.6-4.5 $\times 10^6$
Hb (g/dL)	8.4 \pm 1.36	8.95 \pm 1.58	11.0-15.0
HCT (%)	23.8 \pm 3.65	26.5 \pm 4.65	33.0-46.0
MCV (fl)	74.58 \pm 3.91	76.74 \pm 5.06	80-90
MCH (pg)	26.5 \pm 1.99	25.98 \pm 2.20	27-31.2
MCHC (g/dl)	35.5 \pm 1.54	33.81 \pm 1.04	31.8-35.4
RDW (%)	15.38 \pm 3	14.98 \pm 3.60	11.5-14.5
WBC(cell/ μ L)	8519 \pm 3681	8234 \pm 3052	4-11 $\times 10^3$
Neutro. (%)	49.35 \pm 8.3	48.54 \pm 8.37	40-75%
Lymph.(%)	38.35 \pm 8	38.23 \pm 7.74	15-45%
Mono (%)	7.27 \pm 1.95	7.83 \pm 1.85	5-15%
Eosino. (%)	3.86 \pm 2.58	4.24 \pm 2.18	0-2%
Baso.(%)	1.18 \pm 0.46	1.15 \pm 0.39	0-1%
Platelet.(cell/ μ L)	226 \pm 104	270 \pm 83	150-450 $\times 10^3$
MPV (fl)	6.7 \pm 1.6	7.1 \pm 1.43	6.90-10.6

References

1. Shazia Q., Mohammad Z.H., Taibur Rahman, and Hossain Uddin Shekhar (2012). Correlation of Oxidative Stress with Serum Trace Element Levels and Antioxidant Enzyme Status in Beta Thalassemia Major Patients: A Review of the Literature. Hindawi Publishing Corporation-Anemia. 7
2. Raghuveer P., Vidya P, and Prabhu R.S., "Iron overload in beta Thalassemia, A review," (2009) Journal of Bioscience and Technology, 1 (1): 20–31.
3. Rahul A Ghone, K M Kumbar, A N Suryakar, R V Katkam and N G Joshi (2008) "Oxidative stress and disturbance in antioxidant balance in beta thalassemia major" Indian Journal of Clinical Biochemistry, 23 (4): 337-340.
4. Hallberg, L., Brune, M. & Rossander L. (1986). Effect of ascorbic acid on iron absorption from different types of meals. Studies with ascorbic-acid-rich foods and synthetic ascorbic acid given in different amounts with different meals. Hum. Nutr J: Appl. Nutr. 40: 97-113.
5. Ballot D, Baynes RD, Bothwell TH, et al. (1987). The effects of fruit juices and fruits on the absorption of iron from a rice meal. Br J Nutr. 331–343.
6. Cook, J.D. & Monsen, E.R. Vitamin C, (1977). The common cold and iron absorption. Am. J. Clin. Nutr. 30: 235-241.
7. Institute of Medicine. Food and Nutrition Board. (2000). Dietary Reference Intakes for Vitamin C, Vitamin E, Selenium, and Carotenoids Washington, DC: National Academy Press,.
8. Jacob RA, Sotoudeh G. (2002). Vitamin C function and status in chronic disease. Nutr Clin Care; 5: 66-74.
9. O'Brien RT. (1974). Ascorbic acid enhancement of desferrioxamin-induced urinary iron excretion in thalassemia major. Ann NY Acad Sci 232: 221–225.
10. Tietz Textbook of Clinical Chemistry and Molecular Diagnostics, analytic-vitamin C-4th chapter 6th edition (2008).
11. Mayo Clinic: Mayo Medical Laboratories. (2012). Accessed April 18, <http://www.mayomedicallaboratories.com/test-catalog>
12. Harrison's Principles of Internal Medicine. 17th Edition (2008) pp.2432.

13. Padayatty SJ, Katz A, Wang Y, Eck P, Kwon O, Lee JH, Chen S, Corpe C, Dutta A, Dutta SK, Levine M. (2003). "Vitamin C as an antioxidant: Evaluation of its Role in Disease Prevention". *J Am Coll Nutr* February; 22 (1): 457-464.
14. Chapman R W , Hussain M A , Gorman A , Laulicht M , Politis D, Flynn D M , Sherlock S , and Hoffbrand A V. (1982). Effect of ascorbic acid deficiency on serum ferritin concentration in patients with beta-thalassaemia major and iron overload. *J Clin Pathol.*; 35(5): 487–491.
15. Ray M., Marwaha R. K., Sethuraman G. and Trehan A. (1999). "Scurvy in transfusion dependent beta-thalassemia," *Indian Pediatrics*; 36 (5); 504–506.
16. Susan Claster, John C. Wood, and Thomas D. Coates (2009). Nutritional deficiencies in iron overloaded patients with hemoglobinopathies *Am.J Hematology*.
17. Laila M. Sherief, Sanaa M. Abd El-Salam, Naglaa M. Kamal, Osama El-safy, Mohamed A.A. Almalky, Seham F. Azab, Hemat M. Morsy, and Amal F. Gharieb. (2014). Nutritional Biomarkers in Children and Adolescents with Beta-Thalassemia-Major: An Egyptian Center Experience *BioMed Research International* Volume, 7.
18. Kent Chen, Jung Suh, AnitraC. Carr, JasonD. Morrow, John Zeind, Balz Frei. (2000). Vitamin C suppresses oxidative lipid damage in vivo, even in the presence of iron overload *American Journal of Physiology-Endocrinology and Metabolism* 279; 6.
19. Berger TM, Polidori MC, Dabbagh A, Evans PJ, Halliwell B, Morrow JD, Roberts LJ, Frei B. (1997). Antioxidant activity of vitamin C in iron-overloaded human plasma. *J Biol Chem* 272: 15656–15660.
20. Dissayabuttra T, Tosukhowong P, Seksan P, (2005). The Benefits of Vitamin C and Vitamin E in Children with beta-Thalassemia with High Oxidative Stress, *J Med Assoc Thai*; 88: 317-321.
21. Fischer R, Harmatz PR. (2009). *Hematology Am Soc Hematol Educ Program*; 215-221.
22. Sharma DC, Mathur R. (1995). Correction of anemia and iron deficiency in vegetarians by administration of

- ascorbic acid. *Indian J Physiol Pharmacol.* 39 (4): 403-406.
23. Siegenberg, D. (1991). Ascorbic acid prevents the dose-dependent inhibitory effects of polyphenols and phytates on nonheme-iron absorption. *Am. J. Clin. Nutr.*, 53: 537-541.
 24. Seshadri S, Shah A, Bhade S. (1985). Haematologic response of anaemic preschool children to ascorbic acid supplementation. *Hum Nutr Appl Nutr.* 39 (2): 151-154.
 25. Das N, Chowdhury TD, Chattopadhyay A, Datta. (2004). Attenuation of oxidation stress-induced changes in thalassemic erythrocytes by Vitamin E. *Pol J Pharmacol*; 56: 85-96.
 26. Cighetti G., Duca L, Bortone L, Sala S, Nava I, Fiorelli G and Cappellini M.D (2002). Malondialdehyde in β -thalassaemia patients. *European Journal of Clinical Investigation.* 32; 55–60.
 27. Aust S.D., L.A. Morehouse, C.E. Thomas, (1985). Role of metals in oxygen radical reactions. *J Free Radicals Biol. Med.*; 1; 23–25.
 28. Han-Yao Huang, Lawrence J Appel, Kevin D Croft, Edgar R Miller III, Trevor A Mori, and Ian B Puddey (2002). Effects of vitamin C and vitamin E on in vivo lipid peroxidation: Results of a Randomized Controlled Trial. *American Society for Clinical Nutrition.*
 29. Kashinakunti SV, Kollur P, Kallaganada GS, Rangappa M, Ingin JB. (2011). Comparative study of serum MDA and vitamin C levels in non-smokers, chronic smokers and chronic smokers with acute myocardial infarction in men. *J Res Med Sci.* 16(8): 993-998.