Estimation of malondialdehyde (MDA) levels and the relationship with homocysteine and serum copper in beta-thalassemia patients

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الخلاصة

صممت الدراسة على أساس تقييم العلاقة بين ارتفاع الحامض ألأميني الهموسستين وعنصر نحاس المصل مع المالوندای الديهايد عند الأطفال الذين يعانون من فقر دم البحر المتوسط. اخذت عينات الدم من قسم الثلاسميا في المستشفيات الحكومية. وقد تضمنت الدراسة أختيار خمسون مريضاً يعانون من مرض الثلاسميا وخمسون أصحاء ووجدنا زيادة معنوية لبيروكسيد الشحوم المقاس كمالونداي الديهايد كونه سائد لمرضى الثلاسميا ($p<0.01$) . كان معدل مستوى هموسستين المصلي لمرضى الثلاسميا أعلى من الاصحاء ($p<0.02$) . بالاضافة الى ذلك كان معدل مستوى النحاس المصلي لمرضى الثلاسميا أعلى من الاصحاء($p<0.03$). علاوة على ذلك لوحظ أرتباط موجب بين أرتفاع الحامض الأميني الهموسستين وعنصر النحاس مع مستوى المالوندای الديهايد في مجموعة المرضى ولم يلاحظ في مجموعة السيطرة

Abstract

The study was designed to evaluate the medical relevance of hyperhomocysteinemia and copper levels with malondialdehyde in beta-thalassemia patients . Blood samples were taken from thalassemia departments of a ministry of healthy hospitals. Fifty children with thalassemia (study group) and fifty healthy controls (group control). We found that significantly increased lipid peroxidation, measured as MDA, was demonstrated in thalassemia ($p< 0.01$). The mean plasma homocysteine (tHcy) level in children with thalassemia was significantly higher than the control group($p< 0.02$) In addition, the mean serum copper level in thalassemia patients was significantly higher than the control group ($p< 0.03$). Moreover, a positive correlation was also observed between homocysteine, copper with MDA levels in the patient group but not in the control group.

Introduction

Beta thalassemia major is the most prevalent type of thalassemia as it is common in certain populations. It produces severe anemia in its homozygous state (1). About 190 million people throughout the world have genetic mutations associated with different hemoglobinopathies and more than 90 million of them carry defective genes leading to thalassemia (2, 3).

The free radical field is a large, multidisciplinary research area (4-6). For example, the basic chemistry of superoxide (O_2^-) and hydroxyl (HO^-) radicals was determined many years ago by radiation chemists; the outline mechanism of lipid peroxidation was elucidated by scientists at the British Rubber Producers Association; combustion is a free radical reaction; and some of the most detailed chemical work on peroxidation and antioxidants has been carried out in the food industry and by polymer scientists. In 1954, Gershman and Gilbert proposed that most of the damaging effects of elevated O_2 concentrations on living organisms could be attributed to the formation of free radicals (6).

Oxygen radicals and other activated oxygen species are produced by most, if not all, cells. It is generally believed that membrane lipids are major targets for cellular damage induced by oxygen radicals. Considerable progress has been made in elucidating the effects of oxygen radicals on lipid peroxidation through *in vitro* studies with defined lipid mixtures and *in vivo* studies in normal and pathological states (7-8).In contrast, the chemical effects of oxygen radicals on cell proteins and the biological consequences of such reactions have not been extensively studied and are still poorly understood.

Earlier studies have shown that, in thalassemia there is excess production of reactive oxygen intermediates, such as superoxide anion (O_2^-), hydroxyl radical (OH^-), singlet oxygen and hydrogen peroxide (H_2O_2) within the erythrocytes, all these events lead to oxidative stress. This oxidative stress and a possible consequential accelerated apoptosis may contribute to shortened life span of erythrocytes. Malondialdehyde (MDA), a product of lipid peroxidation is generated in excess amounts in supporting the fact that large amount of membrane bound iron is present in thalassemic erythrocytes (2,9)

Oxidation of LDL is a free radical process in which the polyunsaturated fatty acids contained in the LDL are degraded by a lipid peroxidation process to a great variety of aldehydes (eg, malondialdehyde (MDA). In a cell- free system , LDL can be oxidized by traces of transition metal ions: particularly effective are Cu^{+2} ions, copper is an essential trace element, which is distributed through out the body (10). Besides forming the essential redox-active center in a variety of metalloproteins, such as ceruloplasmin, Cu,Zn Superoxide dismutase, cytochrome C oxidase, dopamine β-hydroxylase, tyrosinase, lysyl oxidase, and ascorbate oxidase (10), reduction of Cu^{+2} to Cu^{+1} may play a role in lipid peroxidation . Cu^{+2} –reduction factors like lipid hydroperoxides($Cu^{+2} + LOOH \longrightarrow Cu^{+} + LOO^{\cdot} + H^{+}$)(11,12).

Homocysteine regulate plasma ceruloplasmin redox state and copper transport into cells . Recently it has been reported that homocysteine (Hcy) can reduce Cu^{+2} to Cu^{+1} and this reaction could potential the cell-damaging property of copper ions to endothelial neural cells in presence of Hcy ($Cu^{+2} + RSH \quad Cu^{+} + 1/2RSSR^{\cdot} + H^{+}$) (12)

Therefore, the present study was undertaken to evaluated the association between MDA (a marker of oxidative stress) with homocysteine levels and serum copper in thalassemia patients

Materials and methods:

Fifty children with thalassemia were taken from thalassemia departments of a ministry of healthy hospitals. All mean age of patients were 6-11 years. There were 50 healthy volunteers (7-12 years) used as control subjects. They were clinically diagnosed on the basis of severe anemia and haemoglobin electrophoresis. Before sampling collection, if any gradients regarding medication taken by patients that would interfere with homocysteine test such as methotrexate, folic acid, or exposed hours ago to nitrous oxide were considered.

Fasting blood samples (10 mL) were collected from patients and controls. The blood samples were centrifuged at 3000 rpm for 10 min at 4^{o}C both the patients and controls' sera were stored at 4^{o}C in an ice chest for no longer than 24 h before freezing.

Statistical analysis

The results are expressed as mean ± SD (1SD). Statistical analysis was performed using student's *t*-test; *p* values <0.05 were considered significant.

Materials

The entire chemicals were imported from BDH Co and Sigma chemical co. Expected Kits from Giesse for copper and zinc.

Assessment of the lipid peroxidation activity:

The assessment of lipid peroxidation process is achieved via determination the byproduct; Malondialdehyde(13).

The level of serum malondialdehyde was determined by a modified procedure described by Guidet B. and Shah S.V.(14). In brief; to 150 µl serum sample add the followings: 1 ml trichloroacetic acid 17.5 %, 1ml of 0.6% thiobarbituric acid, mixed well by vortex, incubate it in boiling water bath for 15 minutes, then allowed to cool.

Then add 1ml of 70% TCA, and let the mixture to stand at room temperature for 20 minutes, centrifuged at 2000 rpm for 15 minutes, and taken out for scanning spectrophotometrically.

The concentration of malondialdehyde = Absorbance at 532 nm

$$\frac{L \times E_o}{} \quad X\ D.$$

L: light bath (1cm)

$E_{O:}$ extinction coefficient $1.56 \times 10^5\ M^{-1}.\ Cm^{-1}$.

D: dilution factor

Assessment of Hcy in plasma colorimetrically

The Hcy concentration in plasma was measured by a multiscan RC microtiter reader. It was used to measure absorbance on the microtiter plates. The microtiter plates were washed with a well wash 4 washing machines.

the Bio-Rad enzyme-linked immunoassay is a microtiter assay that involved four steps: (a) reduction of Hcy, mixed disulfides, and protein- bound forms of free Hcy by dithiothreitol ; (b) conversion of free Hcy and adenosine to S-adenosylhomocysteine (SAH) by bovine SAH hydrolase; (c) competitive binding of sample SAH and immobilized SAH with monoclonal mouse anti-SAH, and spectophotometric measurement of peroxidase activity after the addition of anti-mouse antibody labeled with horseradish peroxidase(15).

Assessment of serum copper colorimetrically:

Serum copper is measured by a colorimetric method using commercially available kit (Giesse).

Results

Children with thalassemia had significantly higher MDA levels ($p<0.01$) than controls, which suggested the presence of increased oxidative stress as shown in Table (I). The serum Cu level tended to increase in these patients, the mean value of Cu concentration in patients was (182.3+22) µg/dL while the mean value for control was (108.3± 20) µg/dL. Cu level was found to be higher in patient group compared with controls with P value of less than 0.05 as shown in Table (II). The plasma Hcy level tended to increase in these patients, the mean value of Hcy concentration in patients was (8.34 ± 0.15) µmol /L while the mean value for control was (5.51±0.17) µmol /L. Hcy level was found to be higher in patient group compared with controls with P value of less than 0.05 as shown in Table (III). Variation of MDA, copper concentration, and homocysteine level showing enhancement or depression in women with unexplained recurrent miscarriage as shown in figure (1). Both serum MDA and Cu levels were significantly higher than those of the controls ($p<0.05$). As shown in figure (1), a significant positive correlation was found between serum MDA levels and serum Cu levels ($R^2=0.51$, $p<0.05$). Similarly, both serum MDA and plasma Hcy levels were significantly higher than those of the controls ($p<0.05$). As shown in figure (3), a significant positive correlation was found between serum MDA levels and plasma Hcy levels ($R^2= 0.5$, $p<0.05$) and also shown in figure (4), significant positive correlation was found between serum Cu levels and plasma Hcy levels ($R^2= 0.53$, $p<0.05$) .

Table I: Mean malondialdehyde concentration (nmol/ml) of controls group and patients group.

Group	Mean value of MDA conc.	SD*	P<0.05
Controls	1.14	0.18	0.01
Patients	2.38	0.19	

Table II: Mean copper levels (µg/dl) of controls group and patients group.

Group	Mean value of Cu levels	SD	P< 0.05
Controls	108.6	20	0.03
Patients	182.3	22	

Table III: Mean Hcy levels (µmol/L) of controls group and patients group.

Group	Mean value of Hcy levels	SD	P< 0.05
Controls	5.51	0.17	0.02
Patients	8.34	0.15	

SD*= Standard deviation

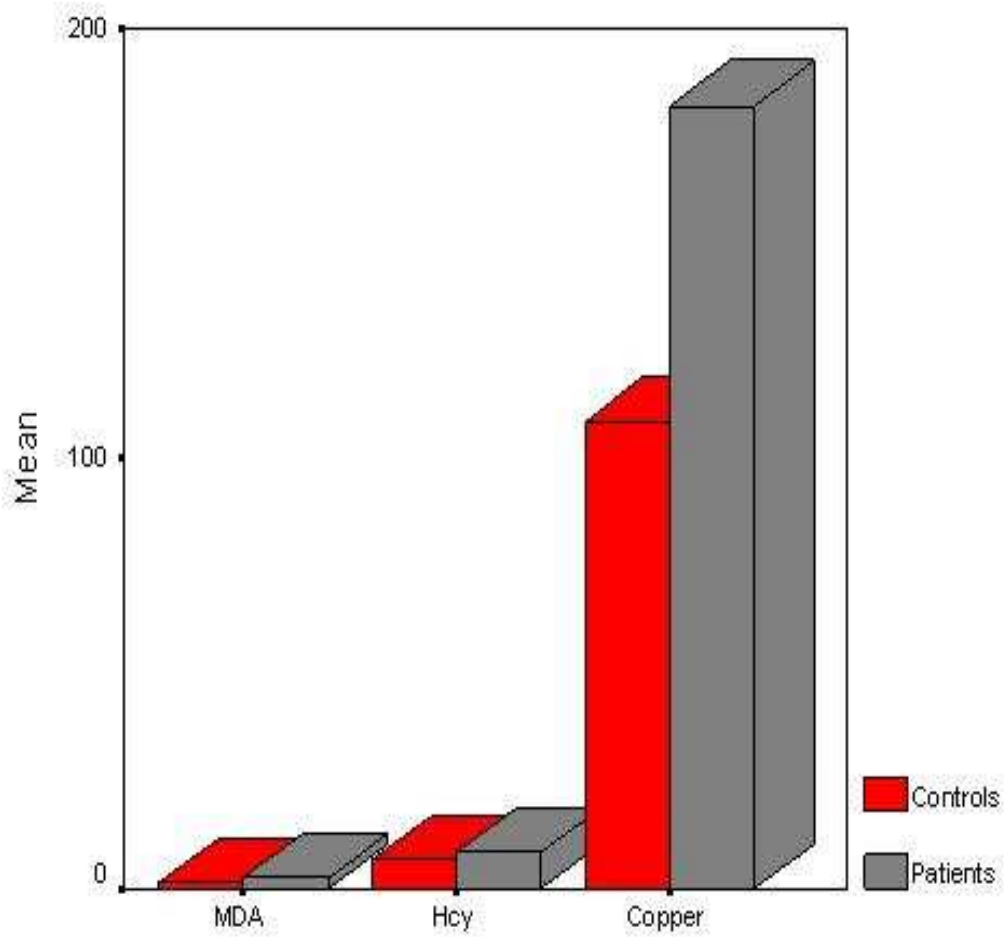

Figure (1). Malondialdehyde (MDA) , copper concentration,and Hcy concentration in children with thalassaemia

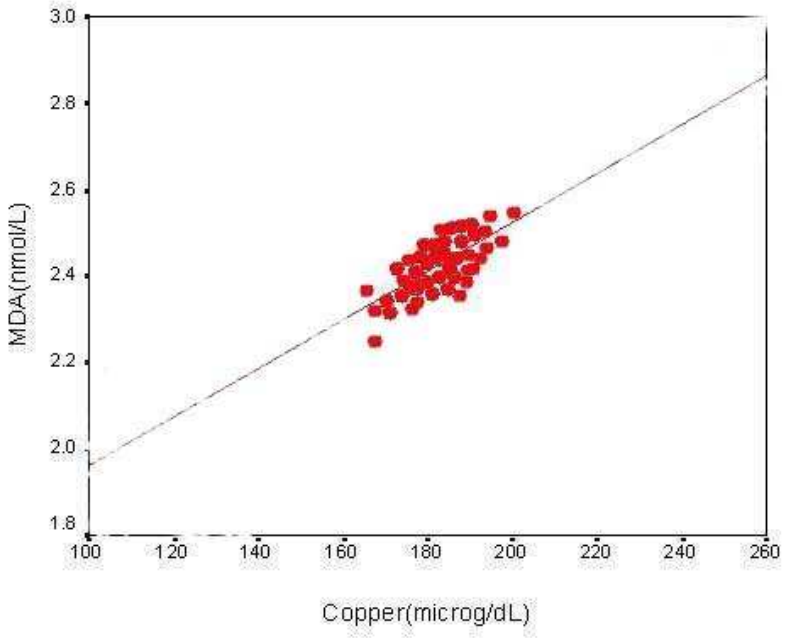

Figure (2). Correlation between serum Cu and MDA levels in children with thalassaemia (n=50,R^2=0.51,P<0.05)

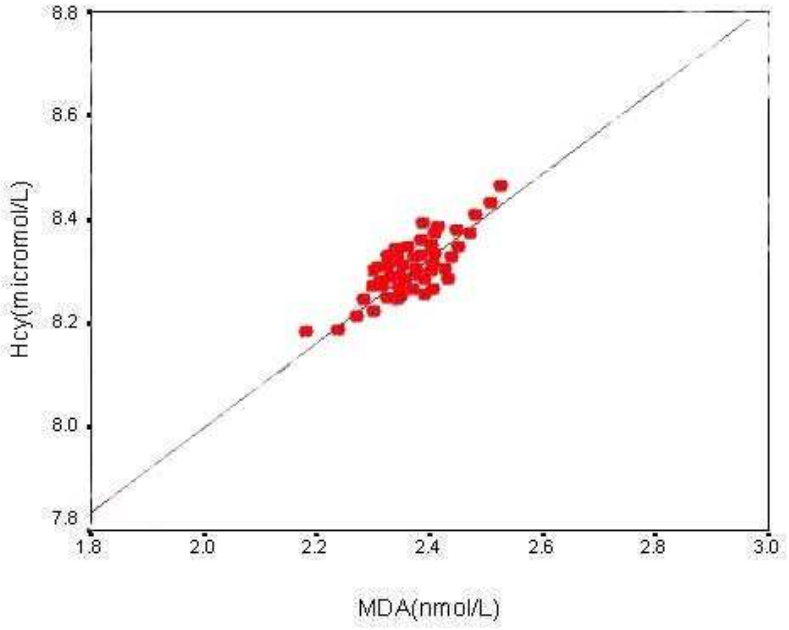

Figure (3). Correlation between plasma Hcy and MDA levels in children with thalassaemia (n=50,R^2=0.5,P<0.05)

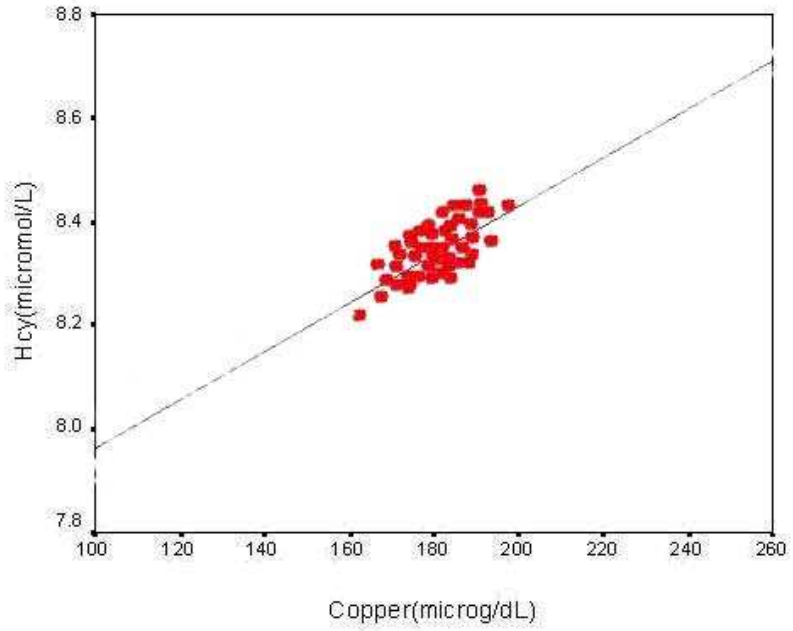

Figure (4). Correlation between plasma Hcy and serum Cu levels in children with thalassaemia (n=50,R^2=0.53,P<0.05)

Discussion

Oxidative stress in cells and tissues usually refers to increased generation of $O_2^{\cdot-}$ and H_2O_2. This can be achieved by activating a large number of phagocytes $O_2^{\cdot-}$and H_20_2 are produced by activated phagocytes and are essential for the killing of many bacterial strains (16), but they can do tissue damage when generated in excess. Lipid peroxidation is a well known example of oxidative damage in cell membranes, lipoproteins, and other lipid-containing structures. (LDL) is an important example of damaging lipid peroxidation that is driven by one-electron LOOH turnover (17). Early studies has investigated the generation of MDA in thalassemic red blood cells, but failed to demonstrate an increase of MDA, unless exogenous peroxidative stress was provided(18).

The suggestion that plasma MDA may be taken into account as a biomarker of oxidative stress in exposed populations has been recently put forward (19) . Giardini et al were able to demonstrate that in thalassemia patients red blood cell MDA was significantly higher as compared with control(20). Naithani et al found that markers of free radical injury such as MDA was significantly elevated in thalassemia compared to control(21). Ghighetti et al found that MDA was higher in the β-thalassemia major (TM) patients than in the untransfused β-thalassemia intermediate (22). This is in agreement with the results of present study in MDA levels are elevated in serum of patient with thalassemia.

Copper participates in the reductive activation of H_2O_2, Causing damage to cellular nucleic acids, proteins and lipids. Interaction of H_2O_2 with O_2 generates more reactive species, such as hydroxyl radicals (4).

In the present study, copper levels were elevated in patient with thalassemia. This is in agreement with the results of other studies(24,25), copper is a common cofactor for many enzymes, and may act as a catalyst in the formation of ROS and the peroxidation of membrane lipid (23). Al-Shamarrai et al found that serum copper was higher in the TM patients than in normal(24) and Bahir et al concluded that the thalassemia

associated with increase copper level and decreased zinc level(25). Sauthipark et al reported that the levels of these trace elements (e.g. copper) in both red cells and plasma were different between the non-thalassemic controls and the disease patients (26). Also, Kajana et al found that The mean serum copper level in patients with thalassemia is higher than the control group(27).

Lipid peroxides decompose under physiological conditions in the presence of copper ions to generate highly cytotoxic aldehydes (28). Of such aldehydes, malondialdehyde (sometimes called MDA) receives the most attention, yet it is now known to be relatively poorly toxic (28). Previous studies have been shown that ability of Cu^{2+} to promote apo B modification has been suggested to reflect differences in the extent of lipid peroxidation , a major mechanism of H_2O_2 toxicity in oxidant stress is the formation of a highly reactive species in the presence of suitable transition metal catalysts(4,29,30,31).Our findings show that a positive correlation was observed between copper level and MDA in patients with thalassemia. This is in agreement with the results of previous studies(4,30,31).

Stamler JW(32) suggested that hyperhomocysteinemia may promote the production of hydroxyl radicals, known lipid peroxidation initiators, through Hcy autooxidation. Cighetti et ai suggested that found the high concentrations of MDA in patients indicated increased membrane lipid peroxidation and potential further oxidation damage(33).Ventura et ai who observed the relationship between Hcy and MDA in hyperhomocysteine patient(34). Ferretti et ai demonstrated that plasma lipoprotein are susceptible to homocsteinylation and interaction between Hcy-thiolactone and amino groups of apo-B lysyl residues of LDLs induces the formation of LDL modified by Hcy-LDL(35). This is in agreement with the results of present study in MDA levels associate with Hcy in serum of patient with thalassemia.

The major finding of the present study is the detection of a strong positive correlation between p-tHcy and copper in patients with thalassemia. This is in agreement with the results of other studies. Emsley et al suggested that superoxide and hydrogen peroxide generation by copper-catalyzed reactions may have participated in the process (36). Starkebanm et al found that Cu^{2+} can result in formation of H_2O_2 during oxidation of homocysteine (37). Mohammad et al concluded that atherogenicity of homocysteine may be related to copper-dependent interaction (38).

Because it was demonstrated that an interaction between copper and homocysteine enhanced the inhibitory action of homocysteine on NO-mediated relaxation of isolated aortic rings of rats. Homocysteine in the presence of a transition metal can mediate oxidation of LDL in vitro. Wall et al suggested a mechanism whereby elevated levels of homocysteine could injure endothelial cells through copper-catalyzed generation of H_2O_2(39). White et al reported that homocysteine (Hcy) can reduce Cu^{2+} to Cu^{+} and this reaction could potentiate the cell-damaging property of copper ions to endothelial and neuronal cells in present of Hcy(40). Reduction of Cu^{2} to Cu may play a role in lipid peroxidation.Cu^{2}-reducing factors like lipid hydroperoxides.

From this point of view, our finding suggested that oxidative stress depend on thalassemia progression may be due, at least in part, to the catalyzation of oxidative stress depend on Hcy levels and Cu levels.

Conclusions

Our finding shows that thalassemia is associated not only with mild or moderate hyperhomocysteinemia but also with increased MDA concentration in the presence of copper. A systematic investigation of factors associated with increased concentrations of p-tHcy, the correlation of homocysteine with copper and the products of peroxidation that may be produced by the homocysteine-copper interaction. We also suggest that

increased oxidative stress present in thalassemia may be resulted from changes in plasma (tHcy) and serum copper levels

References

1-widad NM, Al-Naama L, Meaad. Trace element in patients with beta-thalassemia major. Haem 2003;6:376-83.
2-Das N, Chowdhury TD, Chattopadhyay A, Datta. Attenuation of oxidation stress-induced changes in thalassemic erythrocytes by vitamin E. Pol j Pharmocol 2004;56:85-96.
3-Ambekar SS, Phadke MA, Balpande DN, Moashi GD, Khedkar VA. The prevalence and heterogeneity of beta-thalassemia mutation in the western Maharashtra population : A hospital based study. JHG 2001;1:219-23.
4-Halliwell B, Gutteridge JMC. Free radicals in biology and medicine oxford: Clarendon; 1985.
5-Mccord, J. M.; Fridovich, I. Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). *J. Biol. Chem.* 244: 6049-6055; 1969.
6-Comport M. Lipid peroxidation and cellular damage in toxic liver injury. Lab Invest 1985;53:599-23.
7- Pryor WA. In free radicals in biology 1976, vol 1, 1-99. Academic press, New York.
8-. Tappel AL. (1980) . In free radicals in biology(Pryor WA) ed , vol 4,1-47. Academic press, New York.
9-Livrea MA, Tesoriere L, Pintaudi AM, Calabrse A, Maggio A, Freisleben HJ. Oxidative stress and antioxidant status in beta thalassemia major: Iron overload and depletion of lipid-soluble antioxidants . blood 1996; 88:3608-14.
10-Linder, M. C. Nutritional Biochemistry and Metabolism. New York: Elsevier Science. 1991.
11-Halliwell B and Gutteridge JM. Role of free radicals and catalytic metal ions in human disease: an overview. *Methods Enzymol* 1990;186: 1–85.
12- Burkitt M.J. *Arch. Biochem. Biophys.* 2001**;394**: 117–135.
13-Lunec J., Review Article, Ann. Biochem., 1990, 27, 173.
14-Guidet B., and Shah S.V., Am. J. Physiol., 1989;257(26),F 440.
15-Ebba N, Frode E, Per M U. "Evaluation of novel assays in clinical chemistry: quantification of plasma total homocysteine ".Clin Chem; 2000, vol.46: pp 1150-1156.
16-Babior B.M. Oxidants from phagocytes agents of defense and destruction. Blood, 1984; 64: 959-969.
17-Albert WC. Lipid hydroperoxide generation, turnover, and effectors action in biological systems. J. Lipid Rsea, 1998;39:1529-42.
18-Rachmile EA, Lubin BH,Shohet SB. Lipid membrane peroxidation in beta-thalassemia major. Blood, 1976; 47: 495-505.
19-Niesen F, Mikkelsen BB, Nielsen JB, Anderson HR, Grandjean P. Plasma MDA as marker for oxidative stress: reference interval and effects of life-style factors. Clin Chem, 1997;43:1209
20-Giardini O, Cuntani A, Donfrancesco A, Ruberto U, Lubrano R. Biochemical and clinical effects of vitamin E administration in homozygous beta-thalassemia. Acta vitamin of enzymol, 1985; 7:55.
21-Naithani R, Ghandra J, Bhattacharjee J, Verma P, Narayan S. Peroxidative stress and antioxidant enzymes in children with beta-thalassemia major. Pediatr. Blood cancer, 2006;46:780-5.
22-Gighetti G, Duea L, Bortana L, Sala S, Cappellini MD.Oxidative status and malonialdhyde in Beta-thalassemia patient. Eur J Clin invest, 2002;32:155-60.

23-Chan PC, Peller OG, Kesner L. Copper (II)- catalyzed lipid peroxidation liposomes and erythrocyte membranes. Lipid ,1982;17:331-7.
24-Al-Shamarrai AH, Adaay MH, Al-Tikriti KA, and Al-Anzy MM. Evaluation of some essential element levels in thalassemia major patients in mosul district Iraq. Saudi Med J, 2008; 29:94-7.
25-Bashir NA. Serum Zinc and copper levels in sickle cell anemia and beta-thalassemia in north Jordan. Ann Trop Paediator, 1995;5:291-3.
26-Sauthipark KU, Likidlilid A, Fucharoen S, Pootrakul P, Shumumsirivath D, Ongajyooth S. Red cell and plasma calcium, copper and zinc in beta-thalassemia /hemoglobin E. Southeast Asian J Trop Med Public Health, 1991;22:171-5.
27- Kajana S, Tata T, Sasanakul W, Chuansumrit A. Zinc and copper status of thalassemia children. Southeast Asian J Trop Med Public Heath, 1997;28:877-80.
28- Diny AH, Chan PC, Singlet oxygen in copper catalyzed lipid peroxidation in erythrocyte membranes. Lipid, 1984;19:278-84.
29-Steinbrecher UP. Oxidation of human low density lipoprotein results in derivation of lysine residues of apolipprotein B by lipid peroxide decomposition product. J Biol Chem, 1987;262:3603-08.
30-Arabi M, Alaedioi MA. Metal-ion mediated oxidative stress in the gill homogenate of rain bow trout : antioxidant potential of manganese, selenium, and albumin.Biol Trace Elem Res.2005; 108:155-68.
31-Tikly M, Channa K, Theodorou P, and Gutumian M. Lipid peroxidation and trace elements in systemic sclerosis. Clin Rheumatal . 2005;25;320-4.
32-Stamler JS, Osborne JA, Jareki O, Rabbani LE, Mullins M, and Single D. Adverse vascular affects of homocysteine are modulated by endothelium- derived relaxing factors and related oxides of aitrogen. J Clin Invest., 1993;91:308-18.
33-Cighetti G, Dediasi S, Paroni R, and Allevi P. Free and total malondialdyehyde assessment in biological matrices by gas chromatography- mass spectrometry- what is needed for an accurate detection. Anal Biochem, 1999;266:222-9.
34-Ventura P, Pannini R, Verlato C, Scarpetta G,and Salviolo G. Peroxidation indices and total antioxidant capacity in plasma during hyperhomocysteinemia induced methionine oral loading. Metab Clin Exp, 2000;49;225-8.
35- Ferretti G, Bacchetti T, Maroni C, and Curatola G.Effect of homocysteine of low density lipoproteins on lipid peroxidation of human endothelial cells. J Cell Biochem, 1999;92:351-360.
36-Emsley AM,Jeremy JY, Gomes GN, and Angelini. Investigation of the inhibitory effects of homocyteine and copper on nitric oxide – mediated relaxation of rat isolated aorta. Br J Pharmacol, 1999;126:1034-40.
37-Starkebanm G, Harlan JM, Endothelial cell injury due to copper- catalyzed hydrogen peroxide generation from homocysteine. J Clin Invest, 1986;77:1370-76.
38-Mohammad A, Claes B, Steve JH, and Peter HE. Correlation between plasma total homocysteine and copper in patients with peripheral vascular disease. Clin Chem, 2000; 46: 385-91.
39-Wall RT, Harlan JM, Harker LA, and Rofilio G. Homocysteine- induced endothelial cell injury in vitro: a model for the study of vascular injury. Thromb Res, 1980;18:113-121.
40- White AR, Huang X, Joboling MF, and Barrow CJ. J Neuro Chem, 2001;76:1509-20.