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Evaluation of Certain Inflammatory Markers in Transfusion Dependent β-Thalassemic Patients

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Abstract :

To determine the serum concentration of certain inflammatory markers including TNF-α and hs-CRPβ-thalassemic patients on different types of treatmentand to investigate the possible correlation between these inflammatory markers and iron overload referred to by serum ferritin concentration, moreoverto assesswhether inflammation in β-thalassemia could be controlled by deferasirox or deferoxamine as compared to transfusion dependent patients without iron chelator. Ninety transfusion dependent β-thalassemic children with age range 13–75 months were included in this study, and 30 age and sex matched healthy subjects served as control,thepatients were divided into three groups according to the type of the treatment,each group included 30 patients Group1 comprised β-thalassemic patients without iron chelator, Group2 comprisedβ-thalassemic patients on deferasirox iron chelator while Group3 comprisedβ-thalassemic patients on deferoxamine iron chelator. Serum ferritin, hs-CRP, and TNF-α was measured for all participants by ELISA method. **C**ompared with the control, the serum level of ferritin and hs-CRP were significantly elevated in all patients groups while the serum level of TNF-α were only significantly elevated in Group1 and Group3. Further analysis of the results revealed positive correlation between serum ferritin withTNF-α and with hs-CRP.A comparison among the patients groups show that patients on deferasirox (Group2) had significantly low level of TNF-α as compared to Group1 and Group3. On the other hand serum ferritin and hs-CRP concentration in Group3 were significantly higher than the other two groups, furthermore when Group3 patients are subdivided according to their compliance on deferoxaminethe result show that the serum level of ferritin, hs-CRP and TNF-α are significantly higher in poor compliance subgroup as compared to good compliancepatients and when good compliance subgroup are compared with Group2 no significant deference in the measured parameter was found. **C**hronic inflammatory state is present in these patients withincreased levels of inflammatory markers such as TNF-α andCRP. The observed correlations of ferritin with inflammatory markers imply that iron overload may play a key role in release of these markers. Chelation Therapy with deferasirox can attenuate inflammation and reduces inflammatory markers level while deferoxamine appear to be less effective as most patients suffer from poor compliance on this type of iron chelator..

Introduction

β-thalassemia are inherited defects in the rate of synthesis of β globin chains of hemoglobin, that are widely distributed throughout the world, with considerable frequencies in the Eastern Mediterranean countries, including Iraq.[1,2] Most β-thalassaemias are due to single point mutations, results in either reduced or absent β globin chain synthesis.[3]The excess of unmatched α-globins precipitates in the red cell precursors, damaging the membrane and leading to their premature destruction in the bone marrow. This ineffective erythropoiesis, combined with hemolysis in the periphery and an overall reduction in Hb synthesis (due to decreased β chain production), leads to the marked anemia seen in β-thalassemia.[4]The resultant anemia and other complications can be corrected with repeated regular blood transfusion program;[5] unfortunately, such blood transfusion program will exert its own problems concerning iron overload.[6,7]Each unit of transfused blood contains 200–250mg of iron. As the human body has no mechanism for actively clearing excess iron, a regularly transfused thalassemia patient can quickly develop iron overload after 10–20 transfusions.[6,7]Iron received during blood transfusion is taken up by the reticuloendothelial macrophages and packaged with transferrin for transport throughout the plasma.[8]During situations of iron overload, plasma transferrin proteins become saturated, and the remaining unstored iron circulates through the bloodstream as extracellular non–transferrin-bound iron (NTBI).[8]These NTBI can be taken up into cells, including liver, heart, and endocrine cells, where the iron can participate in the generation of free radicals through Fenton's reaction:[9]

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + HO^{\bullet}$$

These free radicals cause lipid peroxidation and organelle damage that ultimately result in cell death.[9] this event occurs when the cells' normal antioxidant capacity is exceeded; including the storage mechanisms ferritin and hemosiderin.[8] Biomarkers of oxidative stress including plasma malondialdehyde (a marker of lipid peroxidation)[10,11]and plasma protein carbonyls,[12] a marker of oxidation to circulating proteins have been found increased in patients with β-thalassemia with iron overload.[10] Inflammatory biomarkers including C-reactive proteins and cytokines are found to be increased in various inflammatory conditions and have been found useful in studying thalassemia.[10]In patients with iron overload resulting from repeated red cell transfusions, the plasma ferritin concentration is often used to provide an indirect estimate of body iron,[13]serum ferritin levels over 1,000ng/mL indicate excess body iron concentrations;[8]and need of initiating chelation therapy.[14] Sustained levels of over 2500 mg/mL are associated with an increased risk of cardiac toxicity and death in patients with thalassemia.[15,16] There are two goals of iron chelation therapy: the binding of toxic NTBI in the plasma and the removal of iron from the body.[17]Two chelators are approved by the US FDA for use in iron-loading anaemias, parenteral deferoxamine and oral deferasirox,[17] both of which are used in the thalassemia center in Ibn-Al Atheer teaching hospital. Many trials and worldwide clinical experience demonstrate that these drugs can chelate and remove iron, and thereby prevent or improve transfusional heamosiderosis in thalassemia patients.[18]

Patients and Methods

A total of 90 patients of age ranged 13-75 months; all are blood transfusion dependent β-thalassemia patients attending the thalassemia center in Ibn-Al Atheer Teaching Hospital were enrolled in this study, since November 2011 to January 2012. The patients were divided into three groups according to the type of the treatment; ***Group 1*** comprised (30) transfusion dependent β-thalassemic patients who doesn't take iron chelator therapy their age range from 13 to 54 months, ***Group 2:*** comprised (30) transfusion dependent β-thalassemic patients all receiving deferasirox (Exjade®) oral iron chelator at dose 20mg/kg per day, their age range from 28 to 52 months, ***Group 3:*** comprised (30) transfusion dependent β-thalassemia patients receiving deferoxamine (Desferal®) iron-chelating agent at dose range from 20-40 mg/kg per day several times per week, their ages ranged from 32 to 75 months, This group was further subdivided according to the patients' adherence on deferoxamine to good compliance and poor compliance subgroups, patients who took deferoxamine five times per week or more were considered as good compliance subgroup (N=14), while patients who took deferoxamine four times per week or less are referred to as poor compliance subgroup (N=16).

Control

Thirty healthy individuals matched for age and sex with no family history of thalassemia whose age ranged 36-60 months were also included in this study as controls.Subjects were excluded from this study if they had one of the following criteria: a history of hepatitis B or C

infection, diabetes, or cardiovascular disease, recent or active infection at the time of blood sampling.

Methods

Five ml of venous blood was collected in a plain tube from each individual. The tubes are placed in a water bath at 37°C for 15 minutes for blood clotting to occur. Serum samples were obtained by centrifugation of blood at 3000 rpm for 10 minutes the resultant serum was used for the determinations of ferritin, TNF-α, and hs-CRP byenzyme-linked immunosorbent assay. The test kits used for determination of serum ferritin and hs-CRP were manufactured by Monobind Inc. (USA), while TNF-α test kit was manufactured by Assay Max, (USA). Standard statistical methods for the analysis of data were used to determine the mean, standard deviation (SD), unpaired Student's *t* test, ANOVA test, Duncan multiple range test, in addition to Pearson correlation. The statistical results were considered significant at $P \leq 0.05$.[19]

Results

Using unpaired student *t*-test; the results of the present study show that the serum level of ferritin and hs-CRP in Group1, Group2, and Group3 were significantly elevated as compared to control ($p<0.001$). While serum level of TNF-α were significantly elevated in Group1 and Group3 as compared to control ($p<0.001$), but there is no significant difference between Group2 and control ($p>0.05$). (Table: 1, 2, and 3). Using ANOVA test (one way analysis of variance) for studying the difference among the three studied thalassemic groups followed by Duncan test for interpretation of the results,the results of the present study show that there is a significant difference in the serum level of ferritin among the studied thalassemic groups, the serum level of TNF-α in Group2 patients was significantly lower than Group1 and Group3 ($p<0.001$). In the other hand the level of hs-CRP in Group3 were significantly higher than Group1 and Group2 ($p<0.01$), table 4. Good compliance subgroup show significantly low level of serum ferritin, TNF-α and hs-CRP as compared to poor compliance subgroup ($p<0.001$,$p<0.01$, $p<0.01$respectively, table 5).Furthermore there is no significant difference in the serum level of these parameters between good compliance subgroup and Group 2, table (6).

Finally, the relationship obtained from the correlation between serum ferritin level with serum level of hs-CRP and with TNF-αlevel in β-thalassemic patients is a significant positive correlation (hs-CRP: $r=0.359$, $p\leq0.001$, TNF-α: $r=0.275$, $p<0.01$)as seen in figure (1) and (2) respectively.

Note: all results are expressed as Mean±SD, NS= not significant using unpaired t-test.

Table (1):- Comparison of the measured parameter between Group1 and control

Parameter	Group3 N=30	Controls N=30	p-value
Ferritin (ng/ml)	5319±2094.54	85.73±24.36	<0.001
TNF-α (pg/ml)	18.25±7.25	9.06±2.53	<0.001
hs-CRP (µg/ml)	4.7±3.46	0.74±0.28	<0.001

Table (2): Comparison of the measured parameter between Group2 and control

Parameter	Group 2 N=30	Controls N=30	p-value
Ferritin (ng/ml)	3528.9±1473.6	85.73±24.36	<0.001
TNF-α (pg/ml)	10.72±5.13	9.06±2.53	NS
hs-CRP (µg/ml)	2.6±2.91	0.74±0.28	<0.001

Table (3):- Comparison of the measured parameter between Group3 and control

Parameter	Group 2 N=30	Controls N=30	p-value
Ferritin (ng/ml)	5319±2094.54	85.73±24.36	<0.001
TNF-α (pg/ml)	18.25±7.25	9.06±2.53	<0.001
hs-CRP (µg/ml)	4.7±3.46	0.74±0.28	<0.001

Table (4): Comparison of the measured parameters among the studied groups

Parameter	Group 1 N=30	Group 2 N=30	Group 3 N=30	*p*-value
Ferritin (ng/ml)	2388.3±1868.6 a	3528.9±1473.6 b	5319±2094.5 c	<0.001

TNFα (pg/ml)	15.88±8.25 b	10.72±5.13 a	18.25±7.25 b	<0.001
hs-CRP(µg/ml)	2.54±2.57 a	2.6±2.91 a	4.7±3.46 b	<0.01

Among the groups for each parameter, means with different letters horizontally have significant difference at p≤0.05 using Duncan test.

Table (5):- Comparison in parameters between the subgroups of different compliance level to the use of deferoxamine therapy in Group 3

Parameter	**Good compliance N=14**	**Poor compliance N=16**	**p-value**
Ferritin (ng/ml)	3913.79±1530.17	6548.58±1731.15	<0.001
TNF(pg/ml)	14.3±6.44	21.7±6.19	<0.01
hs-CRP (µg/ml)	2.92±2.77	6.25±3.32	<0.01

Table (6): Comparison between good compliance subgroup and Group 2

Parameter	**Good compliance N=14**	**Group 2 N=30**	**p-value**
Ferritin (ng/ml)	3913.79±1530.17	3528.91±1473.69	NS
TNF(pg/ml)	14.3±6.44	10.72±5.13	NS
hs-CRP(µg/ml)	2.92±2.77	2.6±2.91	NS

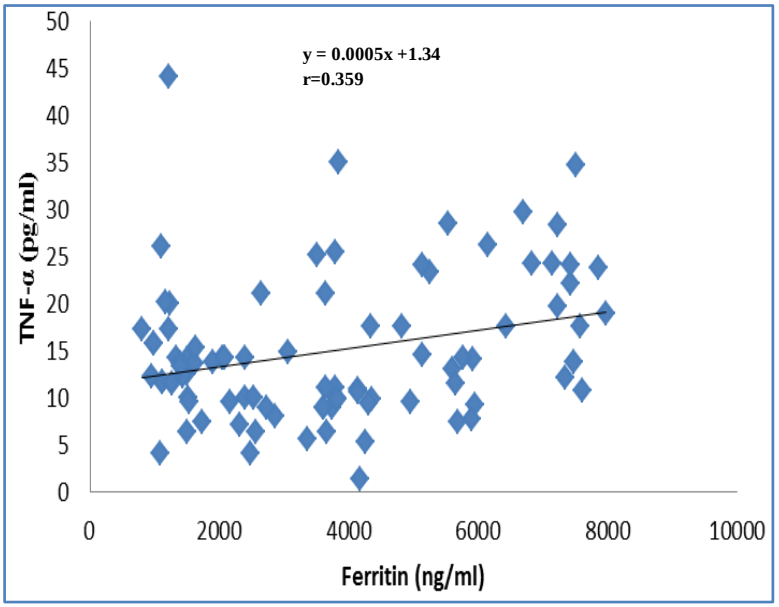

Fig. (1): The relation between ferritin level and hs-CRP

Fig. (2): The relation between ferritin level and TNF-α

Discussion

Blood transfusion and iron chelation therapy has improved treatment of thalassemic patients in the recent years, but iron overload and its toxicities are also common among them. The concept that iron overload leads to increased oxidative stress and inflammation is not new.[20-22]In iron-overloaded states, the binding capacity of transferrin, the major iron transport protein, is surpassed.[23] the resulting increase in NTBIhas been associated with increased reactive oxygen species (ROS). ROS through activation of nuclear factor-κB (NF-κB) lead to enhanced gene expression and production of pro-inflammatory cytokines (such as TNF-α),[24] TNF-α may cause direct injury to compromised cells, facilitating mononuclear cell activation and production of cytokines such as interleukin-6, and up regulating hepatocyte expression of CRP.[25] Furthermore the intracellular storage capacity of ferritin is exceeded and the labile pool of intracellular iron (LPI) is increased. This metabolically active iron catalyzes the formation of free radicals, which can lead to cell damage and eventually death.[21]. In the present study the mean serum ferritin in all β-thalassemic groups were more than 1000ng/ml which point to iron overload, hs-CRP was significantly elevated in all patients groups as compared to control($p<0.001$), (Table: 1, 2, and 3). TNF-α was elevated in Group1 and Group3 as compared to control ($p<0.001$), (Table: 1 and 3), and significant positive correlation was found between serum ferritin with hs-CRP ($r=0.359$, $p<0.001$)Fig.(1),and with TNF-α($r=0.275$, $p<0.01$) Fig. (2). Many studies support such findings; Tsay*et.al* (2010) generated an iron-overloaded mouse by injecting iron dextran into C57/BL6 mice for 2 months. Compared with the placebo group, Iron-overloaded mice had

increased reactive oxygen species and elevated serum TNF-α concentration that correlated with severity of iron overload.[26]Morabito*et.al* (2007), showed an increased circulating levels of TNF-α in β-thalassemic patients as compared to control (P<0.0001) the authors suggesting that heavy transfusional programs were contributed to these results.[20] Regarding hs-CRP; Jokhio*et.al* (2009), found High mean hs-CRP level in β-thalassemic patients (2.52±1.37µg/ml) with a positive correlation with ferritin (r=0.837, p<0.0001), and they concluded that the high CRP in these patients indicate ongoing iron overload toxicity related damage in these patients, progression of cardiovascular diseases and as indicator of morbidity and mortality.[22]Furthermore in Awadallah*et.al* (2011) study the hs-CRP was significantly higher in β-thalassemia major patients than in controls (2.5±1.8µg/ml vs. 1.57±1.5µg/ml, p<0.001) with positive correlation with ferritin (r=0.342, p<0.01).[27] The results of the present study also revealed that β-thalassemic patients on deferasirox iron chelator (Group 2) had significantly lower level of TNF-α than patients without iron chelator (Group 1), (p<0.001),table (4), and there is no significant difference in the serum level of this cytokine between this group and control (p>0.05), table (2). This is due to the ability of the deferasirox to provide sustained reduction in toxic labile plasma iron LPI(chelatable form of NTBI) levels in these heavily iron-overloaded patients, providing 24-h protection from LPI. Deferasirox may therefore reduce unregulated tissue iron loading and prevent further end-organ damage.[28,29] Lower levels of either NTBI or LPI might be expected to diminish iron-induced oxidative injury and possible stress to circulating monocytes and cells of the reticuloendothelial system. Reduced oxidant-stress has been shown to lower monocyte IL-6 and TNF-α release in other inflammatory disease models.[30]Voskaridou *et.al* (2010), suggests that deferasirox is an effective chelator in thalassemia major and significantly reduce TNF-α (p≤0.01) in totalof 52 β-thalassemic patients after 12 months of therapy.[31] In the other hand, patients on deferoxamine iron chelator (Group3) had significantly higher level of TNF-α and hs-CRP than Group2 (p<0.001, p<0.01 respectively), table (4). These finding can be explained byinability of deferoxamine to constantly control levels of LPI because of their short plasma half-lives,[32] as compared to deferasirox which exert its effects for a much longer time after dosing.[28]It is possible that the longer chelator half-life may lower inflammation by better controlling levels of NTBI after chelator administration and the sustained presence of an iron chelator in the plasma may help avoid accumulation of excess iron, thereby preventing iron-related morbidity and mortality.[33]However the poor compliance of patients on the rigorous treatment regimenof deferoxamine therapy appears to be basic reason, because the patients with good compliance on deferoxamine have no significant difference in the serum level of ferritin, hs-CRP and TNF-α than Group 2, table (6), and have significantly low levelsof ferritin, hs-CRP and TNF-αas compared to poor compliance patients,(p<0.001, p<0.01, p<0.01 respectively), table (5). It seems that the benefits of good compliance on deferoxamine over the poor compliance were not only to lower serum ferritin but also reducing the marker of inflammation that elevated in poor compliance patients. This indicates that both iron chelators are effective in reducing these markers if the patient complied well with the treatment. Gharagozloo *et. al* (2009), evaluate the immunologic abnormalities of 28 Iranian

β-thalassemia major patients on deferoxamine iron chelator; the researchers exclude any patients with poor deferoxamine compliance from the study (i.e. patients missed more than one infusion), the result show that there is no significant difference in the serum level of TNF-α between patients and control ($p>0.05$).[34]However the level of these marker appear to be lower in Group 2 especially the level of TNF-α in which the difference between the two compared groups tend toward significant ($p=0.057$). Perhaps because deferasirox take the advantage of long duration of action as compared to deferoxamine, table (6). The high hs-CRP level in Group 3 as compared to Group 1 despite of iron chelator in the former group may explained by the high uncontrolled serum ferritin in Group 3 as compared to Group 1 (5319±2094.54 vs. 2388.34±1868.68 ng/ml, $p<0.001$). In conclusion: A chronic inflammatory state is present in these patients, with increased level of markers of inflammation. The observed correlations of ferritin with TNF-α and hs-CRP imply that iron overload may play a key role in release of these markers in thalassemic patients. Chelation therapy with Deferasirox can attenuate inflammation and reduce inflammatory markers level while deferoxamine appear to be less effective as most patients suffer from poor compliance on this type of iron chelator.

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