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Association of haptoglobin phenotypes with ceruloplasmin ferroxidase activity in β-thalassemia major

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ABSTRACT

Background: Haptoglobin (Hp) and ceruloplasmin (CP) are 2 plasma antioxidants playing a role in preventing iron-induced oxidative damage. This study presents data related to Hp phenotypes and ceruloplasmin ferroxidase activity in relation to iron store markers in patients with β-thalassemia major.

Methods: Blood specimens were collected from 196 subjects (124 β-thalassemia major patients and 72 healthy controls). Serum levels of iron, total iron binding capacity (TIBC), ferritin, high sensitivity C-reactive protein (hs-CRP), ceruloplasmin, and ferroxidase activity were determined using conventional methods. Haptoglobin phenotypes were determined by polyacrylamide gel electrophoresis.

Results: As expected, the mean levels of iron store markers, except TIBC, were significantly higher in patients than in controls. Ceruloplasmin concentrations (mg/dl) and its ferroxidase activity (U/l) were significantly higher in patients than in controls (57.9±18.8 vs 46.9±14.2 and 159.9±47.8 vs 95.3±20.9; p<0.001, for CP and Hp, respectively). As for Hp phenotypes, no significant differences were observed between iron store markers and ferroxidase activity among the control group. In the patients group however, significantly higher concentrations of ceruloplasmin and its ferroxidase activity were observed among patients with Hp2-2 phenotype as compared to patients with the other phenotypes. Additionally, correlations according to Hp phenotypes revealed strong association between ceruloplasmin ferroxidase activity and serum ferritin in patients with Hp 2-2 phenotype and not in the others (r=0.331, p<0.05).

Conclusion: Thalassemia patients with Hp 2-2 phenotype are under greater iron-driven oxidative stress than patients with other phenotypes.
iron from tissue stores and plasma onto transferrin [17,18]. The
importance of CP in iron metabolism was clearly demonstrated by
studies conducted on humans as well as animals that were deficient of
ceruloplasmin [18–23]. These studies demonstrated massive iron
deposition in a number of organs including brain and liver [18–20].
Further studies have demonstrated that CP enhances the efflux of iron
out of hepatocytes [17,23], and that CP administered to CP knock-out
mice can mobilize iron out of liver restoring normal iron homeostasis
[22]. Collectively, these studies suggest that CP is required for efficient
iron release from cells and tissues.

Haptoglobin (Hp) is a polymorphic α2-glycoprotein that exists in
three main phenotypes (Hp 1-1, Hp 2-1, and Hp 2-2) possessing
different structural and functional properties [24]. In circulation, the
main function of Hp is to bind with free hemoglobin (Hb) preventing
loss of iron and kidney damage. It is thus considered a powerful
antioxidant as a result of its ability to prevent hemoglobin-driven
oxidative damage [24]. Many studies however, had demonstrated that
the antioxidative property of Hp and the rate of clearance of free Hb
from circulation are phenotype-dependent [25]. It has been demon-
strated that Hp 2-2 phenotype has lower hemoglobin binding capacity
as compared to the other phenotypes [26,27]. Furthermore, it has been
observed that clearance of Hp 1-1-Hb complex is faster than that of Hp
2-2-Hb complex [25]. Therefore, it was postulated that subjects with Hp
2-2 phenotype are under greater oxidative stress as a result of increased
reodox active iron derived from hemoglobin [28,29]. Once Hp-Hb
complex is formed, it is quickly removed from circulation and scavenged
by the CD163 receptors of hepatocytes and tissue monocytes and
macrophages [30]. Studies have demonstrated that internalization of the
Hp-Hb complex by the CD163 receptors is more potent for the
Hb-Hp2-2 complex than for Hb bound to Hp 1-1 or 2-1 [28,30].
Accordingly, it was suggested that individuals with Hp 2-2 phenotype
are under greater oxidative stress as a result of higher iron accumulation
within monocytes or macrophages [25,28,29].

Taken together, both CP ferroxidase activity and the inheritance
pattern of Hp appear to play a significant role in the homeostasis of iron.
However, data related to the role of these two plasma proteins in
relation to the status of iron store in patients with beta-thalassemia is
very scarce, and not well documented. Therefore, this study was
carried out to evaluate the possible interplay between iron overload on
one hand and ceruloplasmin ferroxidase activity and haptoglobin
phenotypes on the other, in a group of beta-thalassemia major patients.

2. Materials and methods

The study involved 124 beta-thalassemia major patients (69 males and
55 females) and 72 age- and sex-matched healthy controls (39 males
and 33 females). Subjects under age of 8 years were not included in the
study. Patients aged 8 to 30 years (mean 15.8 ± 5.2 years), of whom
only 12 (9.6%) were above 25 years, were recruited from the
thalassemia outpatient clinic at Al Rahma Hospital (Irbid, Jordan).
Clinical history and other relevant data were collected from patients’
files with prior permission of attending physician. All patients received
approximately 350 to 500 ml of packed red blood cells at each
transfusion (3 to 4-week intervals), and all were on iron chelation
therapy using either deferasirox (Exjade) or deferoxamine. Serum
ferritin levels were assessed at least twice a year in thalassemia patients,
and the mean results for the last 5 years were used for evaluation.
Thirty-nine patients were hepatitis C virus (HCV) positive, and 29 had
splenectomy. Clinical data relevant to cardiac complications, endocrine
disorders, or growth retardation were not documented and not involved
in the study. Healthy controls aged 8 to 30 years (mean 16.2 ± 3.6 years)
were selected from various outpatient clinics at the same hospital. None
of the control subjects were thalassemia minors and none had any
history of blood transfusion, anemia, liver disease, or active inflamma-
tory conditions. All participants provided informed written consent,
which was previously approved by the local research ethics committee.

Blood samples were collected in plain tubes. Thalassemia patients’
blood was collected just before blood transfusion. After clotting, serum
was separated by centrifugation and divided into several aliquots
and kept at −20 °C until they are analyzed. Serum iron, total iron binding
capacity (TIBC), alanine transaminase (ALT), high sensitivity CRP
(Hs-CRP), and ceruloplasmin were determined by automated chemistry
analyzer (Hitachi 912, Roche, Germany). Serum ferritin levels were
determined by immunoassay analyzer (Elecsys, Roche, Germany).
Ferroxidase activity was determined by spectrophotometry using
O-dianisidine dihydrochloride method [31]. Haptoglobin phenotypes
were identified using polyacrylamide gel electrophoresis followed by
peroxidase staining (Fig. 1) [32]. The Hp phenotype frequencies were
directly calculated from the number of individuals expressing the
specific Hp phenotype in both patients and controls. Percent of
transferrin saturation was calculated by dividing serum iron by TIBC.

Data analysis was carried out using the statistical software package
of SPSS ver. 17.0 (SPSS Inc, Chicago, IL). Statistical comparison between
groups was assessed by the Kruskal–Wallis test, and results were
presented as mean ± SD. Spearman’s correlation coefficients were
analyzed to study the association between relevant biochemical
parameters in all investigated groups. Statistical significance was set
at p < 0.05.

3. Results

Table 1 demonstrates the general characteristics of subjects
investigated. As expected, iron store parameters, except TIBC, were
significantly higher in patients than in controls. Additionally, the levels of
CP, ferroxidase, CRP and ALT were also significantly higher in patients
than in controls. No significant differences related to age, gender, and
the distribution haptoglobin phenotypes were observed. Furthermore,
splenectomy and/or HCV did not influence the frequencies of Hp
phenotypes among the patients group. Table 2 shows the laboratory
findings according to Hp phenotypes. Within the control group, no
significant differences were observed in all parameters investigated.
Among the patients’ group however, the levels of CP and ferroxidase
activity were significantly higher in patients with Hp2-2 phenotypes as
compared to those with the other phenotypes (p < 0.05 and < 0.01,
respectively). No significant difference was observed between the other
parameter in relation to Hp phenotype. Spearman’s correlation analysis
was conducted to analyze the correlation between ferritin, ferroxidase,
CP, and CRP among the thalassemia group. In all patients combined,
no significant correlation was observed between ferroxidase and ferritin,
however, significant correlation was observed between ferroxidase
activity with CP and CRP (r = 0.330, p < 0.01 and r = 0.179, p < 0.05,
respectively), and ferritin with CRP (r = 0.342, p < 0.01). Correlations
according to Hp phenotype however, revealed strong correlation of

Fig. 1. Polyacrylamide gel electrophoresis of Hp phenotypes. The gel shows the
electrophoretic patterns of the three Hp phenotypes (Hp 1-1, 2-1, and 2-2).
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Serum iron, ferritin, transferrin saturation, and decreased TIBC, it is very likely that our patients were under oxidative stress too. Under such conditions of disturbed iron metabolism as well as of increased iron-derived oxidative stress, it is thus possible that the functional activity of Cp, which is a major plasma antioxidant, is altered in a way to oppose and counteract the consequences of iron overload. Firstly however, it should be mentioned here that the increased levels of CP observed in our study were not attributed to acute inflammatory condition. This is due to the fact that the levels of CRP were not high enough to indicate an active or acute inflammatory condition. Accordingly, the high serum levels of Cp and ferroxidase activity observed in our patients might be attributed to several iron overload-related mechanisms. First, it is possible that the levels were increased to prevent the formation of reactive oxygen species (ROS) through the Fenton reaction. This is achieved by the antioxidative capacity of Cp through the oxidation of Fe2+ to Fe3+, thus preventing oxidative damage of lipids, proteins and DNA [11]. Second, oxidation of the ferrous form of iron to the ferric form enhances its binding with transferrin and thus its transport to storage and/or utilization sites. Since our results indicated high transferrin saturation percent (>85%), it is also possible that Cp is increased to enhance the binding of iron to substances other than transferrin such as albumin and citrate, thus contributing to the formation of more redox active forms of iron [34]. Third, it is possible that Cp is increased in response to accumulated iron in tissues, thus enhancing efflux of iron into transferrin in plasma. Several studies have provided evidence supporting the function of Cp in mobilization of iron between tissues and plasma [15, 17, 18, 23]. Studies on patients with aceruloplasminemia and hemochromatosis [18]–[20], demonstrated marked accumulation of iron in various tissues and organs such as liver and brain. The accumulated iron was attributed to the lack of ferroxidase activity of Cp that is required for the efflux of iron from tissues to plasma. In addition, studies conducted on animals lacking Cp, demonstrated that administration of Cp restored iron balance in these animals [22]. Fourth, it is possible that Cp levels were increased in response to hypoxia and ineffective erythropoiesis. Results from studies conducted on patients with chronic pulmonary obstructive disease demonstrated significantly increased Cp levels, suggesting an important role of Cp in erythropoiesis under conditions of hypoxia [35]. Such conditions of tissue hypoxia and ineffective erythropoiesis are common features among thalassemic patients.

Our results also demonstrated significant correlations between ferroxidase activity with each of Cp and CRP, and serum ferritin with CRP in the patient group only. The correlation between ferroxidase and Cp is an expected one, however, the correlation between Cp and CRP (r = 0.179, p < 0.05) and ferritin with CRP (r = 0.342, p < 0.001), might be attributed, in part, to a mild acute inflammatory condition in our patients.

Interestingly, findings from this study also demonstrated an existing interplay between Cp and its ferroxidase activity and the Table 1

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Results are mean ± SD or as indicated in table. NS: No significant.

Table 2

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Results are mean ± SD or as indicated in table. NS: No significant.

Thalassemia (124) Controls (72) p value
TIBC, μg/dl 266.4 ± 63.9 325 ± 44.5 0.000
Transferrin saturation, % 108.8 ± 8.9 265 ± 12.2 0.000
Serum ferritin, μg/l 2970 ± 2925 35.0 ± 23.7 0.000

4. Discussion

Results from this study demonstrate for the first time a close association of haptoglobin phenotypes and Cp ferroxidase activity with the iron store status in patients with β-thalassemia major. The results revealed significantly higher serum levels of Cp and its ferroxidase activity in thalassemia patients as compared to healthy controls. Additionally, the levels of Cp and ferroxidase were higher among thalassemic patients with Hp 2-2 phenotype as compared to patients with the other Hp phenotypes. Furthermore, a significant correlation was observed between the levels of Cp and ferroxidase with each of serum ferritin and with the levels of serum ferritin among thalassemic patients with Hp 2-2 phenotype only.

Many previous studies have demonstrated that most beta-thalassemia patients are under greater oxidative stress as evidenced by decreased plasma antioxidant capacity, increased markers of oxidative damage, and most of all, increased serum levels of redox active forms of NTBI and LPI [6–10]. This status of oxidative stress is attributed to the toxicity of iron overload as a consequence of life-long blood transfusions, increased intestinal absorption of iron, and to the greater intravascular hemolysis [33]. Based on the findings of high levels of serum iron, ferritin, transferrin saturation, and decreased TIBC, it is very likely that our patients were under oxidative stress too. Under such conditions of disturbed iron metabolism as well as of increased iron-derived oxidative stress, it is thus possible that the functional activity of Cp, which is a major plasma antioxidant, is altered in a way to oppose and counteract the consequences of iron overload. Firstly however, it should be mentioned here that the increased levels of Cp observed in our study were not attributed to acute inflammatory condition. This is due to the fact that the levels of CRP were not high enough to indicate an active or acute inflammatory condition. Accordingly, the high serum levels of Cp and ferroxidase activity observed in our patients might be attributed to several iron overload-related mechanisms. First, it is possible that the levels were increased to prevent the formation of reactive oxygen species (ROS) through the Fenton reaction. This is achieved by the antioxidative capacity of Cp through the oxidation of Fe2+ to Fe3+, thus preventing oxidative damage of lipids, proteins and DNA [11]. Second, oxidation of the ferrous form of iron to the ferric form enhances its binding with transferrin and thus its transport to storage and/or utilization sites. Since our results indicated high transferrin saturation percent (>85%), it is also possible that Cp is increased to enhance the binding of iron to substances other than transferrin such as albumin and citrate, thus contributing to the formation of more redox active forms of iron [34]. Third, it is possible that Cp is increased in response to accumulated iron in tissues, thus enhancing efflux of iron into transferrin in plasma. Several studies have provided evidence supporting the function of Cp in mobilization of iron between tissues and plasma [15, 17, 18, 23]. Studies on patients with aceruloplasminemia and hemochromatosis [18]–[20], demonstrated marked accumulation of iron in various tissues and organs such as liver and brain. The accumulated iron was attributed to the lack of ferroxidase activity of Cp that is required for the efflux of iron from tissues to plasma. In addition, studies conducted on animals lacking Cp, demonstrated that administration of Cp restored iron balance in these animals [22]. Fourth, it is possible that Cp levels were increased in response to hypoxia and ineffective erythropoiesis. Results from studies conducted on patients with chronic pulmonary obstructive disease demonstrated significantly increased Cp levels, suggesting an important role of Cp in erythropoiesis under conditions of hypoxia [35]. Such conditions of tissue hypoxia and ineffective erythropoiesis are common features among thalassemic patients.

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Interestingly, findings from this study also demonstrated an existing interplay between Cp and its ferroxidase activity and the
In conclusion, results from this study suggest that iron-derived oxidative stress and Cp ferroxidase activity are affected by the Hp 2-2 phenotype in β-thalassemia major. However, since this is the first time such findings were reported and no previous work to compare with, more confirmatory studies are needed to consolidate the findings. Therefore, assessment of such interplay, especially in relation to more specific markers of iron overload such as NTBI, LPI, and intracellular ferritin, may need to be thoroughly investigated.

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