Original Article

Hepcidin Levels in Children with Chronic Liver Disease

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ABSTRACT

Background/Aim: We aimed to analyze serum hepcidin level in children with chronic liver disease (CLD) and its relationship with serum cytokines level, liver function tests, hepatic iron content, and liver fibrosis. **Patients and Methods:** The study included 34 children with CLD, and 15 age- and gender-matched healthy children. Serum hepcidin, ferritin, iron level, interleukin-6 (IL-6), transforming growth factor-β (TGF-β), total oxidant status (TOS), and antioxidant status (TAS) were studied in all patients and in the control group. Liver iron content (LIC) was measured from the liver biopsy specimen. **Results:** Serum ferritin levels were higher in patients with CLD than control group (100.1 ± 98.2 ng/mL vs 50.5 ± 32.2 ng/mL, $P = 0.016$). No significant difference was found in hepcidin levels. Hepcidin levels in children with CLD was positively correlated with ferritin ($r = 0.75$, $P = 0.001$), pediatric end-stage liver disease (PELD) score ($r = 0.56$, $P = 0.001$), TAS ($r = 0.42$, $P = 0.02$), but negatively correlated with albumin level ($r = -0.45$, $P = 0.008$). Transferrin saturation and hepcidin:ferritin ratio were significantly low in patients with severe fibrosis compared with patients with mild/without fibrosis (15.5 ± 5.5 vs 34.3 ± 30.1, $P = 0.017$ and 1 ± 0.5 vs 1.9 ± 1.4, $P = 0.04$, respectively). **Conclusion:** Serum hepcidin levels in children with CLD reflect both liver functions and TAS, and severe fibrosis is associated with low hepcidin:ferritin ratio in children with CLD.

Key Words: Chronic liver disease, ferritin, hepcidin

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The liver has important role in the regulation of iron homeostasis. Primarily, it is one of the major storage sites of iron. Additionally, it produces transferrin, iron carrier glycoprotein in the plasma and hepcidin, the key hormone regulating the systemic iron homeostasis.[1] Studies have shown that the latter one has a central role for mediating the iron homeostasis between the duodenum, liver, and bone marrow.[2,3] Hepcidin binds to ferroportin and causes its internalization and degradation in endolysosomes.[4] The decreased level of ferroportin blocks iron flow into plasma from enterocytes, macrophages, and hepatocytes that cause to decrease in plasma iron concentrations.[2-4] Hepatic hepcidin synthesis is decreased in iron deficiency in order to increase iron absorption. Additionally, hepcidin synthesis is decreased in response to hypoxia and in conditions with increased erythropoiesis.[2-4] Conversely, hepatic hepcidin expression is increased in response to inflammation and systemic infection, especially by inflammatory cytokines, such as IL-6, IL-1, and TNF-α.[5] Hepcidin expression is also increased by bone morphogenetic protein signaling pathway in response to increased iron stores.[6] Furthermore, it was shown that oxidative stress is also important in the regulation of hepatic hepcidin expression in recent studies.[7] These regulatory pathways are protecting the body from harmful effects of iron overload, extracellular proliferating pathogens, and ensure that body iron availability matches iron needs.

Apart from these conditions, it was shown that serum hepcidin levels are correlated with liver iron concentration and liver function. Its level is expected to decrease in patients with cirrhosis due to decreased liver function.[8] It was shown that hepcidin expression was decreased in adult patients with alcoholic liver diseases, chronic hepatitis C and B, and nonalcoholic fatty liver diseases via reactive oxygen species–mediated inhibition pathway.[9] These diseases are accepted as mild forms of acquired iron overload.[9,10] Tan *et al*.[11] showed that serum hepcidin levels are decreased with the increasing fibrosis in patients with chronic liver disease (CLD).

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Anemia is very frequent in cirrhotic children due to gastrointestinal bleeding or nutritional deficiencies. Both anemia and decreased hepatic function may decrease the hepatic hepcidin expression and plasma hepcidin levels in children with CLD. On the other hand, it was shown that the levels of some cytokines such as TNF-α, IL-1, IL-10, and IL-12 are increased in patients with CLD and this elevation may cause to increase in serum hepcidin level.[12] Hepatic iron content, particularly serum hepcidin level in children with CLD may be important for the prognosis especially with end-stage disease, and hepcidin inducers or blockers may be used as a therapeutic agent additionally to other therapies. Therefore, we aimed to analyze the serum hepcidin levels in children with CLD and its relationship with cytokines, liver functions, hepatic iron content, and liver fibrosis.

PATIENTS AND METHODS

This is a prospective study, and included 34 children with newly diagnosed CLD (group 1, 8.9 ± 5.4 years, 15 female), and 15 healthy children without known chronic diseases and with normal laboratory parameters for acute or chronic liver disease (group 2, 8.5 ± 5.5 years, 7 female). Patients with the clinical and laboratory findings of acute liver failure, active systemic infection, with primary or secondary hemochromatosis, with hepatocellular carcinoma or adenoma, renal dysfunction, and received red blood transfusion within 3 months were not included into the study.

Laboratory parameters including liver function tests and diagnostic tests for the specific diseases were analyzed from the peripheral blood in all patients. Additionally, serum hepcidin, ferritin, iron level, iron binding capacity, interleukin-6 (IL-6), transforming growth factor-β (TGF-β), C-reactive protein (CRP), total oxidant status (TOS), and antioxidant status (TAS) were studied in all patients. Same laboratory parameters were obtained in all children in the control group. To avoid diurnal variation, peripheral blood was obtained between 7.30 am and 9.30 am after at least 6 h of fasting.

Peripheral blood samples were put into pyrogen-free tubes and serum was harvested, aliquoted, and stored at −80°C until analysis to study hepcidin, IL-6, TGF-β, TOS, and TAS levels. These parameters were studied by ELISA method (Cusabio Biotech, China for hepcidin and KHC0061, CA, USA for IL-6 and KAC1688/KAC1689, CA, USA for TGF-β). TOS of serum was determined using a novel automated measurement method as previously described.[13] Serum TOS levels were calculated in micromoles of H_2O_2 equivalent/L. TAS of the serum was determined using a novel-automated measurement method, developed by Erel.[14] Serum TAS levels were calculated in millimoles of Trolox equivalent/L. The TOS/TAS ratio was used as the oxidative stress index (OSI). To perform the calculation, the units of TAC, mmol Trolox equivalent/L, was converted to mmol Trolox equivalent/L, and OSI was calculated as follows: OSI = ((TOS, μmol H_2O_2 equivalent/L)/(TAS, μmol Trolox equivalent/L) × 100).

Patients without coagulopathy or with improved coagulopathy after fresh frozen plasma underwent liver biopsy for the diagnostic procedure or histopathological examination of the liver (routine department protocol, not for the study). Biopsy specimen of the patients was re-evaluated by the pathologist for the analyzing liver fibrosis, steatosis, and iron staining. Fibrosis was classified according to modified ISHAK score (0–6) as defined by Ishak *et al*.[15] Severe fibrosis was defined as fibrosis stage ≥4. Fatty changes were classified according to Schwimmer *et al*.[16] Iron staining with Prussian blue was done and scored 0–4 as defined by Nash *et al*.[17] Liver iron content (LIC) was measured from the liver biopsy specimen. Sample was digested with concentrated nitric acid in a microwave digestion apparatus (MARS 5 Microwave System, CEM Corporation, Matthews, NC, USA). The sample digest was diluted, fortified with internal standards, and analyzed using inductively coupled plasma mass spectrometry (ICP-MS, Agilent 7500 Series, Agilent Technologies, Santa Clara, CA, USA).[18]

Informed consent for participating in the study was obtained from parents of all cases and the ethics committee of the Karadeniz Technical University Faculty of Medicine, approved this study.

Data were analyzed using SPSS 16.0 software. Differences between groups were calculated using an independent samples *t*-test for the normally distributed data and the Mann–Whitney *U* test for data not normally distributed. Chi-square test was used for the comparisons of qualitative data. Correlations between variables were calculated using linear regression. Values of $P < 0.05$ were considered significant.

RESULTS

Demographic and clinical findings of the patients and control group are shown in Table 1. No significant difference was found between groups in terms of age and gender. Prevalence of anemia was more frequent in patients with CLD (47% vs. 6.6%, $P < 0.05$). A 64.7% of the patients had metabolic liver disease including Wilson's disease ($n = 12$), tyrosinemia ($n = 4$) and glycogen storage disease ($n = 6$).

Laboratory parameters of the patients and control group are shown in Table 2. No significant difference was found between the patients and control group in terms of serum iron, hepcidin, IL-6, TGF-β, CRP, TAS,

Table 1: Demographic and clinical findings of the patients and control group

Parameters	Patients with CLD (group 1, *n*=34)	Control group (group 2, *n*=15)
Age, year	8.9±5.4	8.5±5.5
Gender (M/F)	19/15	8/7
Anemia, *n* (%)	16 (47)*[a]	1 (6.6)[b]
ALT (IU/L), mean±SD, median	284.4±461.9, 280*[c]	34.6±19.7, 38[d]
Primary diagnosis, *n* (%)		
Metabolic liver disease	22 (64.7)	
Wilson's disease	12	
Tyrosinemia	4	
Glycogen storage disease	6	
Cholestatic liver disease	4 (11.8)	
Biliary atresia	3	
PFIC	1	
Autoimmune hepatitis	3 (8.8)	
Budd-Chiari syndrome	1 (2.9)	
Cryptogenic cirrhosis	4 (11.8)	
Child-Pugh score (A, B, C) (%)	61.7, 23.5, 14.8	
PELD score, median (range)	6 (−8 to 43)	
Portal hypertension, *n* (%)	6 (17.6)	
Encephalopathy, *n* (%)	3 (8.8)	
Ascites, *n* (%)	4 (11.8)	
Cholestasis, *n* (%)	10 (29.4)	

*P^{a-d} < 0.05. ALT: Alanine transaminase, PELD: Pediatric end-stage liver disease, PFIC: Progressive familial intrahepatic cholestasis

Table 2: Laboratory parameters of the patients and the control group

Parameters	Patients with CLD (group 1, *n*=34)	Control group (group 2, *n*=15)
Hemoglobin levels, mg/dL	8.9±3.2*[a]	12.9±1.1[b]
Serum iron, µg/dL	77.1±50.3	81.1±34
Transferrin saturation (%)	29.1±27.7	29.4±13.9
IL-6, pg/mL	235.9±42.1	218.5±38.2
TGF-β, ng/mL	19.6±10	17.9±5
CRP, mg/dL	0.8±0.7	1.3±1.1
TAS, mmol Trolox Equiv/L	22.9±16.4	20±12.1
TOS, µmol H_2O_2 Equiv/L	8.7±9.9	6.6±3.3
OSI	87.8±124.9	55.6±63.3
Ferritin, ng/ml, mean±SD	100.1±98.2[c]	50.5±32.2[d]
median (range)	73.3 (6.5-404)	41.7 (15-110)
Hepcidin, ng/mL, mean±SD	125.2±91.5	93.9±61
median (range)	107.8 (10-319.8)	78.8 (7.8-211.5)
Hepcidin:ferritin	1.6±1.2	1.8±0.6

*P^{a-d} < 0.05. SD: Standard deviation, CLD: Chronic liver disease, TGF-β: Transforming growth factor beta, CRP: C-reactive protein, TAS: Total anti-oxidant status, TOS: Total oxidant status, OSI: Oxidative stress index

TOS, OSI, and hepcidin:ferritin levels. Only ferritin levels were higher in patients with CLD than the control group (100.1 ± 98.2 ng/mL vs 50.5 ± 32.2 ng/mL, $P = 0.016$). No correlation was found between serum hepcidin levels and age within the groups.

We found that serum hepcidin levels in children with CLD was positively correlated with ferritin ($r = 0.75$, $P = 0.001$), pediatric end-stage liver disease (PELD) score ($r = 0.56$, $P = 0.001$), and TAS levels ($r = 0.42$, $P = 0.02$), but negatively correlated with albumin level ($r = -0.45$, $P = 0.008$) [Figure 1a–c]. Serum ferritin levels were also positively correlated with PELD score ($r = 0.65$, $P = 0.001$). No correlation was found between hepcidin and other parameters such as TOS, OSI, IL-6, TGF-β, and CRP. No significant difference was found in hepcidin levels between patients with ($n = 16$) and without anemia ($n = 18$), and patients with ($n = 10$) and without cholestasis ($n = 24$). Patients with Child–Pugh score A ($n = 21$) had lower levels of hepcidin and ferritin than the patients with Child–Pugh score B + C ($n = 10$) (99.2 ± 79.3 vs 187.5 ± 92.3 ng/mL, $P = 0.019$ for ferritin and 60.5 ± 46.5 vs 195.1 ± 125.2 ng/mL, $P = 0.008$ for hepcidin) [Figure 2] but had higher hepcidin:ferritin ratio (1.9 ± 1.3 vs. 1.1 ± 0.5, $P = 0.03$).

Histopathological examination could be performed in 25 patients (7 had coagulopathy, 2 did not give permission for the liver biopsy). Histological analysis revealed severe fibrosis (fibrosis stage ≥4) in 24% of the patients (6 of 25 patients) and mild fibrosis (fibrosis stage >0 to <4) in 56% of the patients (14 of 25 patients). Five patients (20%) did not have fibrosis. Steatosis was found in 32% of the patients (8 of the 25 patients). Four percent of the patients (1 of 25 patients) had positive iron staining. LIC (mean ± SD (range, median)) of the 25 patients were 87.9 ± 72 (23.1–308, 65) mg/g. No significant difference was found in terms of hepcidin, ferritin, and hepcidin:ferritin ratio between the patients who underwent and did not undergo liver biopsy.

Transferrin saturation and hepcidin:ferritin ratio were significantly low in patients with severe fibrosis compared with patients mild/without fibrosis (15.5 ± 5.5 vs 34.3 ± 30.1, $P = 0.017$ and 1 ± 0.5 vs 1.9 ± 1.4, $P = 0.04$, respectively). Figure 3 shows the hepcidin:ferritin ratio in healthy children, patients with mild/without fibrosis and severe fibrosis. No significant difference was found in terms of hepcidin, ferritin, and hepcidin:ferritin ratio in patients with or without steatosis.

No correlation was found between LIC and hepcidin, ferritin, hepcidin:ferritin ratio, transferrin saturation, TAS, TOS, OSI, IL-6, and TGF-β.

DISCUSSION

In this study, we found that (1) children with CLD had high ferritin levels compared with healthy children, (2) serum hepcidin level in children with CLD is positively

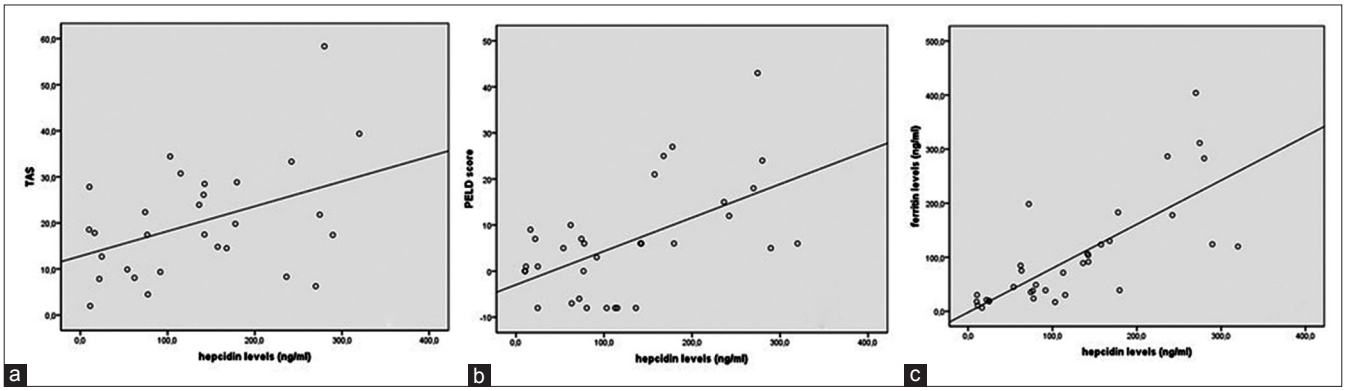

Figure 1: (a–c) Correlation of serum hepcidin levels with total antioxidant status (TAS), pediatric end-stage liver disease (PELD) score, and serum ferritin levels in children with chronic liver disease. Serum hepcidin level was positively correlated with TAS levels (r = 0.42, P = 0.02), PELD score (r = 0.56, P = 0.001), and serum ferritin level (r = 0.75, P = 0.001)

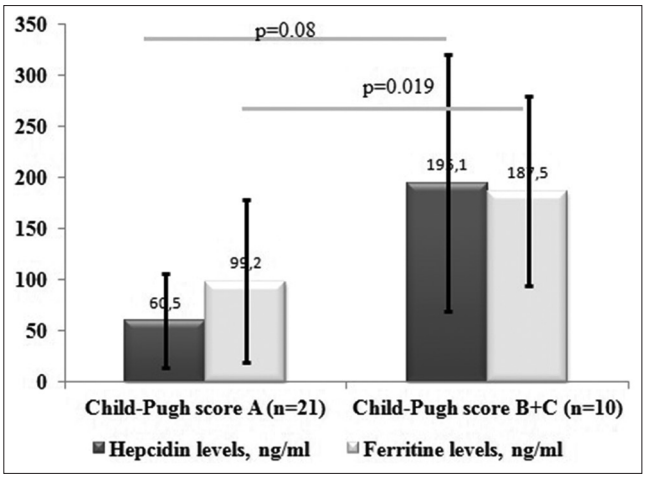

Figure 2: Comparison of the hepcidin and ferritin levels according to Child–Pugh scores in patients with chronic liver disease. Both of them were high in patients with high Child–Pugh score (B + C) (195.1 ± 125.2 vs 60.5 ± 46.5 ng/mL, P = 0.008 for hepcidin and 187.5 ± 92.3 vs 99.2 ± 79.3 ng/mL, P = 0.019 for ferritin)

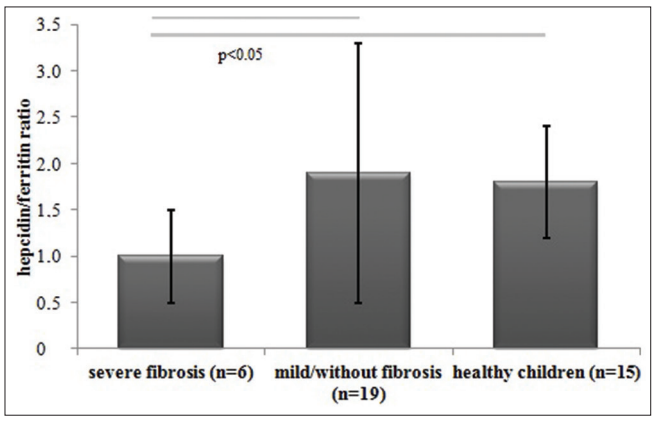

Figure 3: Hepcidin: ferritin ratio in healthy children, patients with mild/without fibrosis, and patients with severe fibrosis. Hepcidin: ferritin ratio was significantly low in patients with severe fibrosis than both patients with mild/without fibrosis and healthy children (1 ± 0.5, 1.9 ± 1.4, and 1.8 ± 0.6, respectively, and P < 0.05 for both patients with severe fibrosis vs mild/without fibrosis and severe fibrosis vs healthy children)

correlated with PELD score, ferritin levels, and TAS but negatively correlated with serum albumin levels, and (3) low hepcidin:ferritin ratio is a potential marker of advanced liver disease (patients with Child–Pugh score B or C, and patients with severe fibrosis) in children with CLD.

In recent adult studies, it was shown that patients with Child C cirrhosis had higher ferritin levels than patients with Child B and A cirrhosis and healthy controls.[19] High serum ferritin level predicts the liver disease-related complications and mortality in patients with cirrhosis awaiting liver transplantation.[20] Furthermore, serum ferritin levels are also good predictors of post-transplant graft and patient survival. Weismuller *et al*.[21] showed that high serum ferritin concentration (>365 μg/L) combined with low transferrin saturation (<55%) predict post-transplant mortality. Elevated serum ferritin levels in these patients may be associated with iron overload in extrahepatic sites, and especially myocardial iron deposition may compromise post-transplant survival in these patients. Serum ferritin levels in patients with CLD may be increased due to (1) intercurrent systemic or local infection, (2) increased body and hepatic iron stores, (3) malignancy, and (4) increased release of ferritin molecules and reduced clearance from circulation.[20] We excluded the patients with the clinical and laboratory findings of systemic or local infection. None of our patients had hepatocellular carcinoma or other malignancy. Additionally, we could not find any correlation with LIC and serum ferritin levels in our patients. Therefore, increased ferritin levels in patients with CLD compared with healthy controls was associated with decreased hepatic functions particularly hepatic necroinflammatory activity because the clearance of serum ferritin is decreased with the reduced liver functions.

Despite most of the patients in our study group had anemia, we could not find any significant difference in serum hepcidin levels between the patients and control group. Similar to our findings, Tan *et al*.[11] found that serum ferritin levels

were high in patients with CLD than the healthy controls, but there was no difference between hepcidin levels. On the contrary, Jaroszewicz *et al.*[22] found decreased prohepcidin levels in patients with CLD. As above-mentioned, serum hepcidin level is expected to decrease in patients with CLD due to decreased hepatic functions. But, hepatic hepcidin expression is regulated not only by hepatic functions but also by local cytokine expression and local reactive oxygen species that may influence the serum hepcidin level oppositely.[1-4] Additionally, etiology of CLD is heterogeneous in our patient group that may affect the serum hepcidin level. Huang *et al.*[23] studied the hepatic expression of hepcidin in children with biliary atresia, and they found that hepatic hepcidin expression is increased in the early stages of the disease but progressively decreased with the advanced disease. Contrary to these findings, they did not find any difference between noncirrhotic and cirrhotic patients secondary to chronic HBV infection. They speculate that hepatic hepcidin expression may be influenced by disease-specific conditions.

We found that serum hepcidin levels are positively correlated with serum ferritin levels, PELD score, and serum TAS, and negatively correlated with serum albumin levels. Contrary to previous studies, we found that serum hepcidin levels increase with the advanced disease.[11,12] But, the increment was more prominent in ferritin levels. Therefore, decreased hepcidin:ferritin ratio may be a more reliable marker of advanced disease. Although the number of the patients with severe fibrosis was small, we found that patients with severe fibrosis had decreased hepcidin:ferritin ratio compared with patients with mild/without fibrosis. Tan *et al.*[11] reported similar findings to our study that hepcidin:ferritin ratio was progressively decreased in patients with progressive fibrosis. In previous studies, it was shown that hepatic hepcidin expression is decreased due to oxidative stress induced by iron, especially in adult patients with chronic HCV infection and alcoholic and nonalcoholic liver diseases.[9,24] Reactive oxidative stress downregulates the hepatic hepcidin transcripts by inhibiting DNA binding activity of the transcriptional factor CCAAT/enhancer binding protein α.[24] Decreased hepcidin expression increases the hepatic iron accumulation, and increased hepatic iron accumulation may cause insulin resistance, fibrogenesis, and mutagenesis.[9] But CLD due to chronic HCV infection, alcoholic and nonalcoholic liver diseases in childhood are very rare, and none of our patients had these etiologies. Most of the patients had metabolic and cholestatic liver disease. We could not make subgroup analysis in each disease etiology group due to small number of patients. We speculate that despite decreased liver functions, other factors such as antioxidants and cytokines may induce hepatic hepcidin expression in order to prevent hepatic or body iron overload in children with CLD.

CONCLUSION

In conclusion, this is the first cross-sectional study about the hepcidin levels in children with CLD. We found that patients with severe fibrosis had decreased hepcidin:ferritin ratio. Hepcidin agonists; hepcidin inducers, such as antioxidants, minihepcidins, genistein, supplements and phlebotomy; iron chelation; or dietary restriction are the novel therapeutic agents in adult patients and under investigation for patients especially with chronic HCV related-cirrhosis, alcoholic cirrhosis, nonalcoholic fatty liver diseases, and hepatocellular carcinoma.[24-26] Further prospective larger studies are needed with homogenous groups in order to analyze the effect of serum hepcidin level on outcome in children with CLD.

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