Importance of Iron and Iron Metabolism in Nonalcoholic Fatty Liver Disease

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Abstract: Iron homeostasis disturbances are associated with liver disease. Non-alcoholic steatohepatitis is part of the spectrum of non-alcoholic fatty liver disease, which can progress to hepatic cirrhosis and end-stage liver disease. Increasing information supports that multiple factors underlie the development and progression of nonalcoholic steatohepatitis. However, the relation between non-alcoholic steatohepatitis and iron metabolism/ overload is still controversial. We review the recent literature, both basic and clinical, regarding iron homeostasis as it pertains to the pathogenesis of nonalcoholic fatty liver disease.

Key Words: Iron, iron overload, insulin resistance, metabolic syndrome, nonalcoholic fatty liver disease.

INTRODUCTION

Iron is an essential micronutrient. The recommended intake is less than 1 mg/day. In mammals, iron is a component of heme in oxygen-transporting and -storage proteins, occurs as iron–sulfur, and is an essential component of proteins with redox and nonredox roles, including enzymes of the mitochondrial respiratory chain and ribonucleotide reductases, which are involved in deoxyribonucleotide synthesis. Iron deficiency is listed by the World Health Organization as one of the top 10 leading risk factors for significant health impairment or death [1]. However, excess iron has several deleterious consequences in that it promotes the generation of reactive oxygen species (ROS) through the Fenton reaction [2]. ROS damage lipid membranes, proteins, and nucleic acids.

Nonalcoholic fatty liver disease (NAFLD) is an increasingly recognized condition that may progress to end-stage liver disease, ranging from steatosis, steatohepatitis, advanced fibrosis, and cirrhosis (in 3% of patients with NAFLD) [3]. The pathological picture resembles that of alcohol-induced liver injury; however, it occurs in patients who do not abuse alcohol [4]. Many pathogenic processes have been described in this disease. One of the most accepted theories of chronic liver damage describes it in terms of 'two hit damage' [5], in which an initial insult is followed by a second insult that triggers the development of a chronic inflammatory state. In this way, changes in liver metabolism predispose the liver to generate highly reactive radicals that perpetuate liver injury. Thus, iron and iron metabolism could play important roles in chronic liver disease. However, clinical data have produced contradictory results [6]. This article analyzes the current information about iron metabolism and its clinical importance in NAFLD.

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AN OVERVIEW OF IRON HOMEOSTASIS

The absorption, use, storage, and export of iron are the main processes involved in iron homeostasis. The more relevant iron intestinal transporters, divalent metal transporter 1 (DMT1) and duodenal cytochrome b, are localized in the apical membrane of the proximal small intestine [7]. DMT1 protein isoforms and mutations alter subcellular trafficking and absorption, respectively [8]. Heme absorbed from the diet is degraded and added to the intracellular nonheme iron pool. For iron to be exported from enterocytes, macrophages, and hepatocytes, it must be oxidized by ferroportin and then bound to transferrin [9], although recent evidence suggests that the ferroxidase activity can be provided by ceruloplasmin or other molecules [10]. Once iron ions are bound to apotransferrin (forming holotransferrin), the iron is internalized *via* the transferrin receptor 1 (TfR1)–transferrin complex. Subsequently, the ferroreductase Steap3 releases the iron into the cytosol and the TfR1–apotransferrin complex is returned to the cell surface. In the cytosol, excess iron can be either stored in ferritin or exported by ferroportin [11].

Precise regulation of iron homeostasis is required to prevent iron overload and its consequences. Iron regulatory proteins (IRPs) are essential components of sensory and regulatory systems [12]. IRP1 and IRP2 recognize specific sequences of iron-responsive elements and regulate ferritin expression [13]. When the intracellular concentration of iron is low, IRPs bind to iron-responsive elements with high affinity, downregulating the expression of ferritin. If a cell is iron-sufficient, IRPs lose their affinity and fail to bind to iron-responsive elements. These mechanisms allow the rapid modification of gene expression in response to fluctuations in iron concentrations, which prevents iron toxicity. How-

1389-5575/08 $55.00+.00

ever, not only total iron levels regulate iron homeostasis mechanisms. Other regulatory factors include: 1) the Fe–S cluster, 2) the function of aconitase, and 3) disturbances in the regulation of IRP1. Many of these additional pathways can be affected by oxidative or inflammatory stresses [14].

Several mechanisms are involved in the regulation of extracellular iron concentrations, and one of the most recently described is hepcidin, a liver-produced protein [15]. Hepcidin-deficient mice exhibit a phenotype similar to that of human hereditary hemochromatosis [16]. Conversely, overexpression of hepcidin-1 causes severe iron-deficiency anemia [17]. Hepcidin forms part of a homeostatic negative feedback system. An increase in serum iron results in the hepatic production of hepcidin, which interacts with ferroportin on the enterocyte membrane to cause internalization of the complex. Iron is trapped intracellularly until the enterocyte is shed into the intestine. As iron continues to be used, serum levels fall, leading to the suppression of hepcidin secretion and strong expression of ferroportin, with iron transfer across the enterocyte basolateral membrane restoring serum iron levels. Hepcidin-induced ferroportin downregulation may explain the trapping of iron in macrophages and hepatic stores in situations associated with increased hepcidin, such as inflammation or infection [18].

IRON-INDUCED CELLULAR DAMAGE

Iron is the most important element of the Fenton/Haber–Weiss reaction. In this reaction, the superoxide radical reduces ferric iron to ferrous iron, which reacts with hydrogen peroxide to produce highly reactive hydroxyl radicals. This phenomenon explains the generation of ROS in several organelles, including mitochondria, peroxisomes, and microsomes. Superoxide dismutase, catalase, glutathione, α-tocopherol, and β-carotene are involved in ROS homeostasis by degrading the latter into nontoxic products, thus controlling intracellular levels [19]. In circumstances in which intracellular iron levels are elevated, the generation of ROS leads to oxidation damage [20].

Lipid peroxidation is mediated by the effects of the hydroxyl radical on the polyunsaturated fatty acids in the lipid membranes, with the consequent production of lipid peroxyl radicals that perpetuate the damage [21].

In iron overload states, iron accumulates in lysosomes and, although this mechanism is considered a protective cellular response [22], disproportionate accumulation leads to lysosomal fragility and dysfunction [23]. In the final pathway, cellular injury is overcome by the liberation of iron and hydrolytic enzymes [21].

Lipid peroxidation participates in several deleterious processes in the cellular machinery [24]. *In vivo* experiments have demonstrated that, at high iron concentrations, the mitochondria undergo depolarization, oxidative phosphorylation is uncoupled, and osmotic swelling occurs [25]. These changes are regulated by mitochondrial permeability transition pores [26] and caspase-activity-induced apoptosis [27].

Finally, overwhelming data indicate that iron induces DNA damage. This damage can be induced by increased levels of etheno-DNA) [28], mutation of the tumor suppressor gene p53, and reactive aldehydes [21].

LIVER DAMAGE

Fibrogenesis is a finely regulated complex phenomenon in which chronic liver injury results in the accumulation of extracellular matrix. The pathways by which iron alters liver homeostasis and predisposes the liver to fibrosis are not well understood. Several *in vitro* studies have demonstrated that isolated hepatic stellate cells from highly iron-loaded rats have an increased capacity to produce collagen [29]. This effect depends on the hepatic iron concentration [30]. This form of hepatic fibrosis has an interesting topographic pattern, beginning in acinar zone 3 and progressing into zone 1. This effect might be explained by differences in vitamin A stores in the liver, oxygen saturation, and glutathione levels [30, 31]. These data support the clinical observation that periportal hepatocytes are preferentially loaded in high-iron states.

Iron overload has an important role in oxidative stress through the generation of the reactive products of lipid peroxidation. These changes could exacerbate the activation of hepatic stellate cells *in vitro* [32]. However, current information suggests that oxidative stress only perpetuates the activation of hepatic stellate cells. This discrepancy could be explained by the fact that hepatic stellate cells express DMT1 and ferroportin-1, both of which are implicated in iron homeostasis [33]. How ferritin and transferrin signals regulate the proteins that are involved in the biology of hepatic stellate cell quiescence has not been investigated.

Another factor that explains liver fibrosis in iron overload diseases is the role of matrix remodeling. Pathological studies have demonstrated that the hepatic iron concentration and hepatic iron index are associated with tissue inhibitor of matrix metalloproteinases (TIMP)-1, but not with the stage of fibrosis [34].

Finally, iron overload is considered to be an inflammatory state, during which interleukin-10 and interferon-γ production is altered. These cytokines are considered important elements in inflammatory and fibrogenic processes [35]. Similar results have been observed for transforming growth factor-β1 and tumor necrosis factor-α [36, 37].

CLINICAL ISSUES IN NAFLD

Several studies have demonstrated an association between iron overload and NAFLD. The greatest epidemiological evidence was derived from the study of Ruhl and Everhart [38], in which data of the Third U.S. National Health and Nutrition Examination Survey (NHANES III) were analyzed. It was shown that, after the exclusion of the common causes of liver disease, the prevalence of abnormal alanine aminotransferase (ALT) levels was 14% in men and 12.2% in women. Serum iron concentrations were higher in subjects with elevated ALT levels than in those with normal ALT levels (98 ± 2.3 *vs* 87 ± 0.7 μg/dL, respectively; $P < 0.001$), confirming an increased risk of elevated ALT through multivariate analysis (OR, 1.13; 95% CI, 1.06-1.21). Furthermore, fasting insulin concentrations correlated negatively with both transferrin saturation and iron concentration (by univariate and multivariate analysis). Fernandez-Real *et al.* have shown a decrease in the development of diabetes mellitus in blood donors, which correlated with a reduction

in iron deposits and an increase in insulin sensitivity [39]. Furthermore, the hepatic extraction and metabolism of insulin decreases with increasing iron stores, leading to hyperinsulinemia [40].

Younossi *et al*. [41] described the clinical and histopathological features of 65 patients to assess the prevalence of hepatic iron overload in patients with NAFLD. Interestingly, these authors demonstrated that significant iron accumulation was not common in these patients. Furthermore, iron was not associated with poor clinical or pathological outcomes.

The lack of a conclusive association was subsequently confirmed. Fargion *et al*. [42] analyzed the *in vivo* and *in vitro* effects of iron on insulin resistance and obtained very interesting results: 1) phlebotomies induce a significant decrease in ALT levels and the homeostasis model assessment of insulin resistance (HOMA-IR) indices, and 2) HepG2 cells cultured in iron-depleted medium displayed twofold increases in ^{125}I-insulin-specific binding and insulin receptor mRNA expression compared with those of HepG2 cells cultured in normal medium. Furthermore, serum ferritin levels correlate with the extent of visceral and subcutaneous fat areas, and hepatic fat content [43]. Currently, the data suggest that iron overload markers are useful in identifying subjects at risk of nonalcoholic steatohepatitis and, consequently, a liver biopsy should be performed [44]. However, Sumida *et al*. [45] investigated whether iron removal by phlebotomy improves serum transaminase activities in patients with biopsy-proven nonalcoholic steatohepatitis. Nine patients underwent phlebotomy biweekly until they approached iron deficiency (serum ferritin concentrations lower than 30 μg/L). The mean serum ferritin levels of these patients fell from 563 to 18 μg/L. The treatment reduced the mean serum ALT activity from 126 to 56 IU/L (P = 0.002). This intervention requires further investigation to clarify its applicability in patients with nonalcoholic steatohepatitis.

Our group described the effects of age, sex, and body mass index (BMI) on elevated serum ferritin (> 300 μg/L for men, > 200 μg/L for women) in 1,757 blood donors from Mexico City [46]. The prevalence of hyperferritinemia was 12% in men and 4.8% in women, and this prevalence increased in parallel with increasing age. In the 50–64 years age group, the prevalence was 29% and 9.6% for men and women, respectively. Regression analysis showed that, in men, there was a significant association between serum ferritin and age, BMI, and recent blood donation (P < 0.01). In women, no association was seen with BMI or recent blood donation.

Mutations of the hemochromatosis gene (*HFE*) have been assessed in patients with NAFLD. Bugianesi *et al*. [47] studied 210 newly diagnosed Italian patients with NAFLD tested for C282Y and H63D HFE mutations using multiple amplification reactions. They found that the prevalence of these mutations did not differ from those of blood donors and did not modify liver iron concentrations, ALT levels, or histological findings.

In a cross-sectional study, we described the lack of association between elevated serum ferritin and *HFE* gene mutations. The prevalence of *HFE* gene mutations was 26.4% and the genotype frequencies for H63D/WT, C282Y/WT, and H63D/H63D among subjects with elevated serum ferritin were 91.6%, 1.8%, and 6.5%, respectively [48].

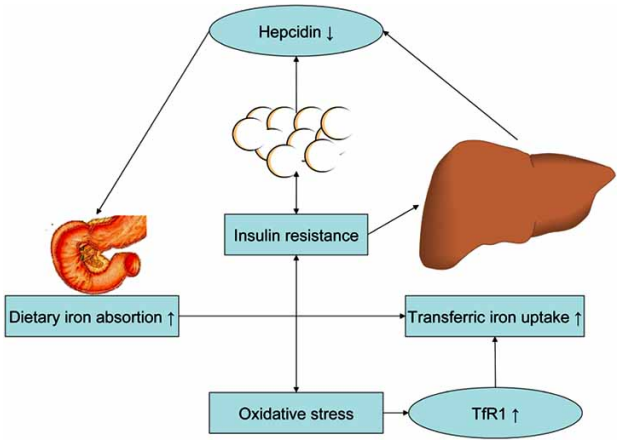

Fig. (1). Association between iron overload states and nonalcoholic fatty liver disease.

Among outpatients referred with elevated serum ferritin (n=482) [49], 119 (25%) had ferritin concentrations greater than 1000 μg/L. HFE-linked hemochromatosis, nonalcoholic steatohepatitis, and alcohol-related liver disease were the top three diagnoses. HFE-linked hemochromatosis accounted for 28%–42% of the diagnoses in all subgroups. The percentage of patients diagnosed with HFE-linked hemochromatosis was similar in the 300–1000 μg/L ferritin group and > 1000 μg/L ferritin group (P = 0.067). Among patients with ferritin levels greater than 1000 μg/L, 63% underwent a liver biopsy. Of those with elevated liver iron concentrations (> 35 mmol/g dry weight), 71% had transferrin saturations greater than 50% (88% of C282Y homozygotes and 43% of non-C282Y homozygotes). The HFE-linked hemochromatosis accounted for less than half of diagnoses in an outpatient population referred for elevated ferritin.

Recently, another element has been assessed that could affect iron metabolism, mutations of α1-antitrypsin (*AAT*). Valenti *et al*. [50] analyzed 353 nonrelated subjects with NAFLD, using DNA amplification by polymerase chain reaction and restriction with *Taq*I, to determine their *AAT* and *HFE* genotypes. In that study, the PiS/wt AAT mutation was more prevalent in subjects with NAFLD than in those without NAFLD (8.8% *vs* 3%, respectively; P = 0.03), confirming an increased risk of NAFLD (OR 3.9; 95% CI 1.2–12.8). Those subjects with *AAT* mutations had higher ferritin levels, especially among males. Although liver iron overload indices were higher in patients carrying *AAT* mutations, no significant difference in the severity of steatosis, inflammation, or liver damage was observed.

A recent study at the Mayo Clinic evaluated features of the metabolic syndrome and NAFLD in subjects with C282Y/C282Y or C282Y/H63D hemochromatosis and iron overload. The metabolic syndrome was present in 27% of subjects, hepatic steatosis in 50%, and steatohepatitis in 21%, with significant fibrosis in 44%. Overall, neither the metabolic syndrome nor any of its components was associ-

ated with significant fibrosis or a higher mean fibrosis stage. C282Y/H63D compound heterozygous individuals with glucose intolerance had more severe fibrosis than did those without glucose intolerance (1.0 ± 1.0 *vs* 0.1 ± 0.3, respectively; $P = 0.01$) [51].

Finally, new clinical information suggests that iron metabolism, obesity, and NAFLD could be associated with inflammatory cytokines (Fig. **1**), considering that all diseases are associated with common long-term low-grade inflammation [52].

CONCLUSION

Iron overload states can influence glucose metabolism and oxidative stress, becoming a cofactor in liver damage. Although clinical information suggests that iron disturbances are not an independent cause of liver damage in subjects with NAFLD, the identification of iron overload is relevant in patients with chronic liver disease irrespective of etiology due to the potential benefit of its correction.

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Received: 05 December, 2006 **Revised: 14 July, 2007** **Accepted: 19 July, 2007**