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Hepcidin gene expression and biochemical changes in high fat diet induced albino rats

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ABSTRACT

The regulation of lipid metabolism is one of the liver's core functions. Fatty liver accumulation, inflammatory process and insulin resistance have been involved in the development and progression of non-alcoholic fatty liver disease (NAFLD), however resulting findings pointed an important role also for iron overload and triglyceride accumulation. In this study, we investigate the relative expression of hepcidin in addition to several biochemical parameters in the onset of steatosis to understand whether its impairment could be an early event of liver inflammatory injury. Sixteen rats were divided into two groups eight per each ,one group fed with normal diet and the other with high fat diet (HFD) for 6 weeks, after which serum lipid profile, transaminases levels, iron and ferritin were evaluated, in addition to hepcidin mRNA quantitation. In HFD fed rats, an increased serum transaminases levels, triglyceride and iron in comparison with the control group, associated with the increase in hepcidin relative expression and ferritin decreased level. In conclusion, the current study suggests that increased exposure to fatty acids subverts hepatic iron metabolism, favouring the induction of iron and hepcidin expression, so, hepcidin may serve as a marker for better diagnosing and monitoring NAFLD in early stages in order to avoid progression of the disease.

Introduction

High level of caloric intake has been associated with many diet-induced complications, including metabolic syndrome, cardiovascular disease and non-alcoholic fatty liver disease (NAFLD) [1]. Non-alcoholic fatty liver disease (NAFLD) encompasses a spectrum of liver disease ranging from simple benign steatosis to non-alcoholic steatohepatitis (NASH) [2,3]. The precise mechanisms of non-alcoholic steatohepatitis (NASH) development are not well understood. Although a so-called "two-hit hypothesis" [4] NASH can also expand in the absence of insulin resistance and simple benign steatosis (*i.e.*, initial hit) [5]. The potential candidates regarded as the "second hit" include oxidative stress, inflammation and transformation in mitochondrial function [6-10]. Iron is also considered as a "second hit" in liver injury [11] and a role for iron has been reported in NASH pathogenesis. Patients with NAFLD/NASH usually display elevated serum iron indices and hepatic iron content [12,13]. Iron disorder is considered one of the serious causes of liver damage [14,15] as its homeostasis is maintained by liver contribution through two major mechanisms: iron storage and hepcidin hormone secretion. Intracellular endoplasmic reticulum (ER) stress induced by nutrient excess has been shown to stimulate hepcidin expression and leads to hypoferremia in mice [16,17].

It has also been shown that mice fed a high-fat diet undergo sustained hepatic inflammation, which influences hepatic hepcidin expression, resulting in a reduction of the hepatic iron level [18]. These reports suggest the possibility that lipid metabolism interacts with iron metabolism. Bile duct ligation was found to reduce the hepatic iron level but pravastatin administration reversibly increased the level, affecting expression of iron metabolism-related genes in the rats [19]. Over nutrition, the central feature of the "modern lifestyle", with carbohydrates, fats or both has a key role in the multiple parallel hits-related to NAFLD. In this study rats fed a high fat diet, representing a model of hepatic steatosis, were used to investigate whether the dysregulation of hepatic iron metabolism in addition to hepcidin expression play a role in the early events of steatosis and inflammation in order to find a relation between impaired iron homeostasis, liver inflammatory damage and ongoing of disease.

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Materials and methods

Animal design

16 albino rats were purchased and housed in the Center of biological experiments and experimental surgery of the faculty of medicine, Cairo university, the rats were divided into two groups:Group I :included 8 rats as control , fed normal diet and group II: included 8 rats fed high fat diet (HFD) rich in saturated and unsaturated fats and low in carbohydrates (60% fats, 20% carbohydrates and 20% proteins) for 6 weeks.

Sample collection

After 6 weeks of high fat diet (HFD), Blood was collected in two ways: In EDTA free tubes, clotted for 30 minutes then centrifuged at 5000 rpm for 10-15 minutes. The serum was collected and kept at -20^{o}C until used for the biochemical analyses, and in tubes with EDTA for RNA extraction from whole blood.

Biochemical tests

Aspartate aminotransferase (AST), alanine aminotransferase (ALT) were carried out using Spectrum Kit (E.C.2.6.1.1 and E.C.2.6.1.2, respectively), Albumin, Urea, Creatinine, iron, Total cholesterol and High-density lipoprotein (HDL) were carried out using Biodiagnostic kits for enzymatic colorimetric method, Low-density lipoprotein cholesterol (LDL-C) was calculated as [total cholesterol – HDL-C-triglyceride x 0.2]. Triglyceride (TG) and glucose were carried out using BioSTC Kit, and ferritin was measured using Ferritin-turbidimetric kit from Linear Chemicals, Spain according to the manufacture's protocol.

Real-time PCR

Total RNA was extracted from whole blood using the QIAamp RNA Mini Kit Quigen, according to the manufacturer's instructions. cDNA was synthesized using a reverse transcription kit (High capacity cDNA Reverse Transcription Kit, applied Biosystems) from 1 ug total RNA. PCRs were performed using ABIPrism 7500 fast real-time PCR system instrument and software (Applied Biosystem).The thermal profile was 10 min at 95°C followed by 40 cycles of two-step PCR denaturation at 95°C for 15 s and annealing/extension at 60°C for 60 s. Each sample contained 1µg cDNA in 10ul 2X Power SYBRGreen PCR Master Mix (Applied Biosystem) and 100 pmole of each primer in a final volume of 20 µl. The analysis of real time PCR output data followed the manufacturer-suggested delta Ct method. Cycle threasholds (Ct) were measured and the relative expression of hepcidin gene was calculated by comparison of Ct values, using one calibrating sample from the control group. All samples were normalized to the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Melt curve analysis was used to confirm the production of a single amplicon for each gene tested. The ΔCt for each sample was determined using the equation ΔCt = Ct target gene – Ct reference gene to calculate the relative expression of each gene to the internal reference control [20].

List of primers used for quantitative real time PCR:

Target Gene	Forward Primer (5′–3′)	Reverse Primer (5′–3′)
GAPDH	AGATCCACAACGGATACATT	TCCCTCAAGATTGTCAGCAA
Hepcidin	GAAGGCAAGATGGCACTAAGCA	TCTCGTCTGTTGCCGGAGATAG

Statistical analysis

Data were analyzed using SPSS (Statistical Program for Social Science version 17). Quantitative variables were described as mean, SD. The NPAR TESTS was used to compare quantitative variables between groups. The Mann–Whitney test was used instead of the unpaired t-test for nonparametric data ,

Spearman correlation coefficient test was used to rank variables positively or negatively.

P values greater than 0.05 were considered insignificant; P values less than 0.05 were considered significant; and P values less than 0.01 were considered highly significant.

Results

Effect of high fat diet on the biochemical parameters

Rats subjected to HFD show no significant difference in comparison with the control group regarding albumin, creatinine, Total cholesterol, HDL, LDL and glucose as P value was more than 0.05 (**Table 1**).

The mean values of serum ALT and AST were significantly higher among HFD group (19.66 ±1.65 and 7.1 ±0.42 respectively) compared with control group (8.38 ±0.63 and 5.06 ±0.76, respectively) with P<0.05, the mean values of Triglyceride (TG), Iron and urea were significantly higher among HFD group (133.38 ±17.98, 264.88 ±40.87 and48.3 ±3.85 respectively) compared with control group (107.63 ± 36.53, 186.88 ±41.69 and 34.29 ±4.97 respectively) as shown in **Table 1**.

Our results show a positive significant correlation between the ALT and iron in HFD group while there is a negative correlation between ALT and ferritin and show a positive significant correlation between the AST and iron in HFD group while there is a negative correlation between AST and ferritin (**Table 2 & Figs. 1&2**).

Effect of High Fat Diet on the Hepcidin expression

The ΔCt for each sample was determined using the equation ΔCt = Ct target gene – Ct reference gene to calculate the relative expression of Hepcidin gene to the internal reference control (GAPDH), the results show a relative expression of Hepcidin in both groups with higher value of ΔCT relative expression in HFD group than the control group (-5.51 ±4.31 and -7.57 ±0.8, respectively).

Table 1: Comparison between the HFD group and control regarding the biochemical parameters.

Parameters	Control group N= 8	HFD group N= 8	Test of significance
ALT (U/L)	8.38 ±0.63	19.66 ±1.65	Z = -3.37 ,P=0.001
AST (U/L)	5.06 ±0.76	7.1 ±0.42	Z = -3.37 ,P=0.001
ALB (g/dl)	4.06 ±0.46	4.38 ±0.16	Z = -1.49 ,P=0.136
Creatinine (mg/dl)	0.64 ±0.03	0.65±0.04	Z = -0.48 ,P=0.635
T.Chol (mg/dl)	63.88 ±12.65	70.5 ±5.61	Z = -0.84 ,P=0.399
TG (mg/dl)	107.63 ±36.53	133.38 ±17.98	Z = -2.42 ,P=0.015
HDL (mg/dl)	11.63 ±1.98	11.66 ±1.53	Z = -0.21 ,P=0.833
LDL (mg/dl)	29.41 ±9.55	34.75 ±6.6	Z = -1.26 ,P=0.207
Iron (umol/L)	186.88 ±41.69	264.88 ±40.87	Z = -2.85 ,P=0.004
Ferritin (ug/L)	6.09 ±0.85	5.06 ±0.37	Z = -2.11 ,P=0.035
Glucose (mg/dl)	111 ±6.66	118.13 ±11.74	Z = -1.58 ,P=0.113
Urea (mg/dl)	34.29 ±4.97	48.3 ±3.85	Z = -3.16 ,P=0.002

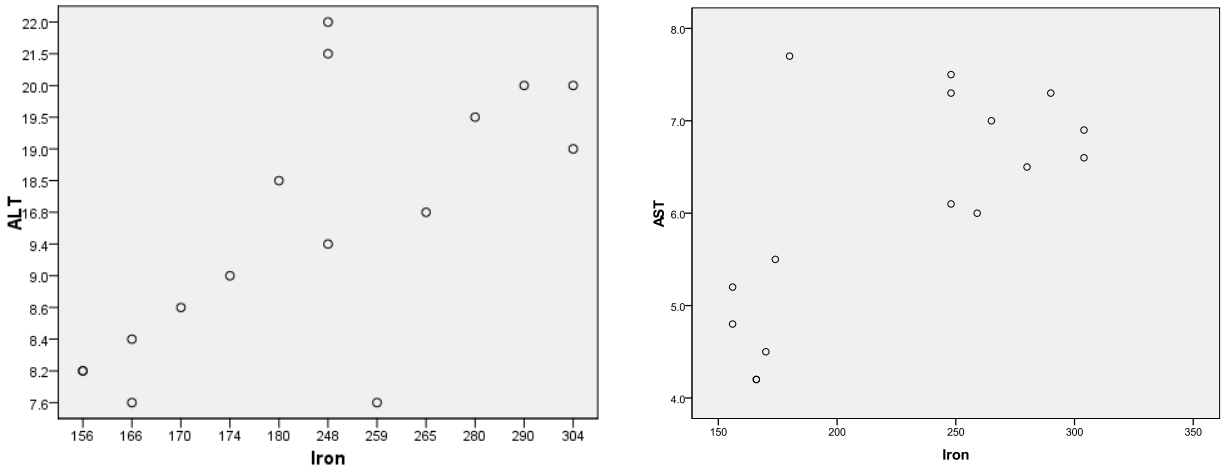

Fig. (1): Correlation between transaminase enzymes (ALT &AST) and Iron.

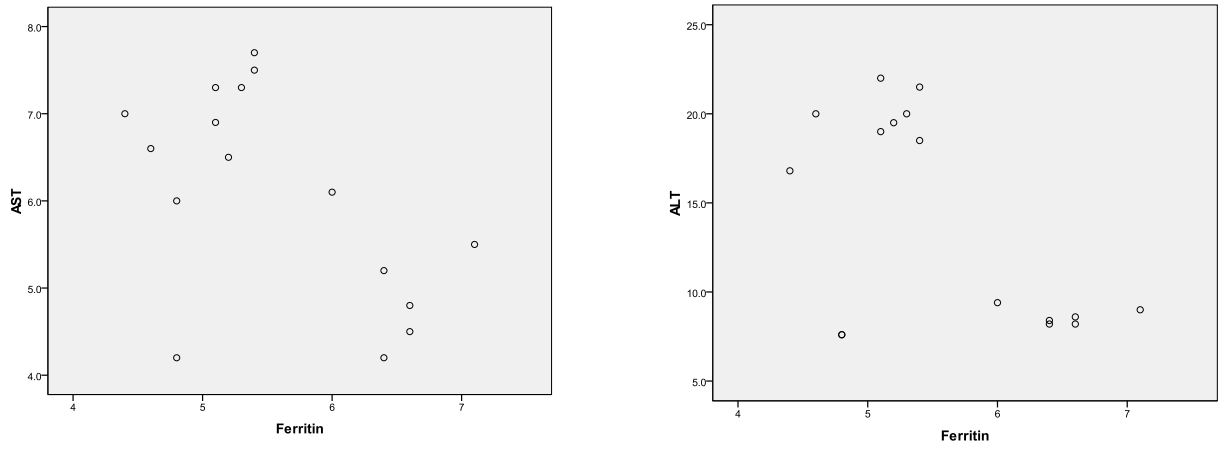

Fig. (2): Correlation between transaminase enzymes (ALT &AST) and Ferritin.

Table 2: Correlation between ALT, AST, iron and both iron and ferritin.

	Iron Sig.(2-tailed)	Ferritin Sig.(2-tailed)
ALT	0.002**	0.023*
AST	0.003**	0.029*
Iron	-	0.006**

*Correlation is significant at 0.05 level (2-tailed)
**Correlation is significant at 0.01 level (2-tailed)

Discussion

Iron overload could induce liver injury [21]. Since hepcidin is the master regulator of iron hemostasis, response of hepcidin to iron change is more sensitive specially when occurs with liver injury. Based on our observation, the HFD group show a significant increase in iron accompanied with a relative hepcidin expression. In contrast, some studies have reported a reduction in hepcidin level in some clinical patients with liver diseases [22-24]. One plausible reason is that the regulation of hepcidin in liver injury is complicated, and may vary according to the nature or severity of the damage for some liver diseases with increased transaminases serum level, such as chronic hepatitis C [24] and alcoholic liver diseases [25]. Among the hepatic enzymes, ALT is most closely related to liver fat accumulation [26]. Our results agree with the findings of previous studies showing the elevated ALT concentration as an independent predictor of incident non alcoholic fatty liver disease (NAFLD) [27]. This finding could be explained by the higher specificity of ALT for liver injury [26]. Also, as a gluconeogenic enzyme [27], increased ALT has been demonstrated to be an indicator of impaired insulin signalling, which might not necessarily be associated with liver injury due to hepatic steatosis [28,29]. In our study the liver function enzymes (AST and ALT) were significantly increased in HFD group as compared with control group meaning that there is a liver injury or inflammation which may be because of the iron-driven free radical production (**Table 1**).

In HFD fed rats group we found a significant increase in the iron as compared with control group (264.88 ±40.87and 186.88 ±41.69 respectively) while there were a significant decrease in ferritin level between the two groups, these findings were proposed of an increased iron uptake into hepatocytes, which could lead to an increase of harmful free-iron since ferritin content was decreased. The limited iron-storage capacity makes the cells susceptible to iron-catalyzed reactive oxygen species (ROS) damage, contributing to the progression of liver damage. As a rule, the increase of intracellular iron decreases Iron regulatory protein-1(IRP1) binding activity, while other factors are able to regulate this activity, including oxidative stress [30]. Moreover, the reduction of ferritin biosynthesis due to the activation of IRP1 by oxidative stress is well documented [30,31], and our results stick to these findings.

However, under conditions of oxidative stress, the early degradation of liver ferritin contributes to expand the intracellular free iron pool that, later on, activates multiple molecular mechanisms to reconstitute ferritin content [32]. Kowdley *et al* [33] examined in human the relationship between elevated serum ferritin and NAFLD severity. They showed that serum ferritin can be considered a marker in identifying patients with NAFLD who are at increased risk of more advanced disease, even among patients without hepatic iron deposition, this is in agreement with our results .Other recent studies report alterations of iron metabolism in HFD fed animals. In a mouse model of diet-induced obesity without inflammation the authors evidenced a decreased serum and tissue iron and an increased serum ferritin while this results are in contrast with our results which show an increase in the iron level and decrease in ferritin level in HFD rats compared with the control group (**Tables 1&2**).In this study an increase in hepatic iron and related oxidative stress together with an increase in the relative expression of hepcidin in addition to triglyceride increased level in HFD group serum may be used as a marker for steatosis. However, many other studies are confirmed with our results evidencing a significant increase in hepcidin associated to inflammation and often to hypoferremia [34,35]. Taken all together, our data clearly show an early impairment of iron metabolism in the initial stage of steatosis contributing to the progression of the disease and the concept of "multiple hits" in the progression of inflammation in NAFLD [36].

Conclusion

The results show that it is useful to use the combination between the increased level of aminotransaminase enzymes in addition to both the iron and hepcidin level as predictive markers of liver inflammation or injury due to fatty diet in order to prevent the prognosis of the disease to NAFLD, and due to the small samples size, a larger randomized study should be carried out in the future.

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