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The importance of iron and copper accumulation in the pathogenesis of non-alcoholic steatohepatitis

Non-alkolik steatohepatit potogenezinde demir ve bakır birikiminin önemi

Kemal AKIN[1], Ali Reşit BEYLER[1], Muhsin KAYA[1], Esra ERDEN[2]

Ankara University Medical School, Departments of Gastroenterology[2], Pathology[2], Ankara

Background/aims: *The pathogenesis of non-alcoholic steatohepatitis (NASH) is poorly understood. Hepatic iron and copper overload can directly cause lipid peroxidation and eventually hepatic damage. The aim of this study was to investigate the role of hepatic iron and copper accumulation in the development of NASH.* ***Methods:*** *Fifty-three patients with NASH were studied. All patients underwent liver biopsy. Clinical and biochemical variables were examined. Serum iron, serum iron binding capacity (SIBC), transferrin saturation, ferritin, ceruloplasmin, and 24-hour urinary copper level were measured. The presence of stainable iron and copper on liver biopsy specimen was investigated.* ***Results:*** *Serum iron level in 14 (26%) patients, SIBC in 2 (3.7%), ferritin level in 1 (1.9%) and transferrin saturation in 2 (3.7%) patients were elevated. One male patient had abnormality in serum iron metabolism showing the possibility ofhemochromatosis. Slightly decreased serum ceruloplasmin level in 4 (7.5%) patients and slightly elevated 24-hour urinary copper amount in 6 (11%) patients were identified. No patients had the abnormality showing the possibility of Wilson disease. NASH was grade I in 25 (47%) patients, grade II in 20 (38%) and grade III in 8 (15%). Fourteen (26%) patients had no fibrosis, 31 (59%) patients had mild fibrosis. None of the patients had bridging or septal fibrosis or cirrhosis. No hepatic iron or copper staining was demonstrated in any patient.* ***Conclusion:*** *There was no correlation between hepatic iron and copper accumulation and the development of NASH.*

Anahtar kelimeler: Iron, copper, non-alcoholic steatohepatitis

Amaç: *Non-alkolik steatohepatitin (NASH) patogenezi tam bilinmemektedir. Karaciğerde aşırı demir ve bakır birikimi direkt lipit peroksidasyonuna ve karaciğerde zedelenmeye yol açabilir. Bu çalışmanın amacı karaciğerde demir ve bakır birikiminin NASH gelişimindeki rolünü araştırmaktı.* ***Yöntem:*** *NASH tanısı almış 53 hasta çalışmaya alındı. Tüm hastalara karaciğer biyopsisi yapıldı. Hastaların klinik ve biyokimyasal özellikleri incelendi. Serum demir (Fe), serum demir bağlama kapasitesi (SDBK), transferrin saturasyonu (TS), ferritin, seruloplazmin, 24 saatlik idrar bakır düzeyi ölçüldü. Karaciğer biyopsisinde boyanabilen demir ve bakır varlığı araştırıldı.* ***Bulgular:*** *Serum Fe seviyesi 14 (%26) hastada, SDBK 2 (% 3,7), ferritin seviyesi 1 (%1,9) ve transferrin seviyesi 2 (% 3,7) hastada artmıştı. Bir erkek hastada hemokromatosis varlığını düşündüren serum Fe metabolizması bozukluğu bulguları vardı. Hafif azalmış serum seruloplazmin seviyesi 4 (%7,5) hastada ve hafif artmış 24 saatlik idrar bakır seviyesi 6 (%11) hastada saptandı. Hiçbir hastada Wilson hastalığını düşündürecek patolojik bulgu saptanmadı. NASH 25 (%47) hastada grade I, 20 (%38) hastada grade II, ve 8 (%15) hastada grade III düzeyindeydi. On dört (%26) hastada fibrosis saptanmadı. Otuz bir (%59) hastada hafif derecede fibrosis vardı. Hiçbir hastada köprüleşme şeklinde veya septal fibrosis veya siroz saptanmadı. Hiçbir hastanın karaciğer biyopsisinde demir veya bakır varlığı saptanmadı.* ***Sonuç:*** *Yaptığımız bu çalışmada karaciğerde demir veya bakır birikimiyle NASH gelişimi arasında herhangi bir ilişki saptanmadı.*

Key words: Demir, bakır, non-alkolik steatohepatit

INTRODUCTION

In 1980, Ludwig et al. coined the term non-alcoholic steatohepatitis (NASH) (1). In some studies, the histologic diagnosis of NASH has hinged solely on the presence of macrovesicular steatosis and lobular inflammation, while others have applied stricter histologic criteria, including macrovesicular steatosis, lobular inflammation, hepatocyte degeneration or ballooning, and/or hepatic fibrosis (2). The disease is considered milder than alcohol-induced disease; most patients were evaluated for abnormal liver function test results and were asymptomatic. In some patients with NASH, disease can develop to progressive liver disease and cirrhosis (3, 4). Early studies described NASH mainly with obesity , type II diabetes mellitus, alcohol abuse, hyperlipidemia, protein malnutrition, acute starvation, and medications (2,5), but the disease is increasingly recognized in patients who lacked these risk factors. The pathogenesis of

Address for correspondence: Muhsin KAYA
SSK Bölge Hastahanesi, Diyarbakır, Turkey
Phone: +90 412 224 07 74-90 532 347 94 58
Fax: +90 412 224 21 38
E-mail: muhsinkaya20@hotmail.com

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NASH is poorly understood. Hepatic triglyceride accumulation occurs when there is a shift in fatty acid metabolism to favor net lipogenesis rather than lipolysis (2). Lipid peroxidation is probably the most important pathogenic mechanism in NASH, but other factors likely contribute to its development. Recent studies suggest that hyperinsulinemia and insulin resistance have a role in the pathogenesis of NASH (6,7).

Accumulation of iron and copper is commonly seen in patients with iron overload or in the later stages of Wilson disease, respectively (8). There is controversial evidence that hepatic iron may play a role in the pathogenesis of NASH. In contrast, Bacon et al. documented abnormal ferritin and/or transferrin saturation, and elevated hepatic iron concentration in NASH (3); Angulo et al. (9), Younossi et al. (5), and Chitturi et al. (10) documented that significant iron accumulation is not seen in most patients with NASH.

Copper overload causes alterations in lysosomal fragility, decreases membrane fluidity, alters fatty acid composition of membranes and catalyzes lipid peroxidation (8,11). There is no information regarding the significance of hepatic copper accumulation in the pathogenesis of NASH.

The aim of this study was to evaluate the importance of copper and iron in the pathogenesis of NASH in the Turkish population.

MATERIALS AND METHODS

Patients

Fifty-three patients in whom NASH was diagnosed were studied. All patients

were prospectively enrolled between January 2000 and January 2001. The pathologic diagnosis of NASH was based on the following criteria: (1) a liver biopsy showing the presence of steatosis (>10%), as well as lobular inflammation and hepatocellular degeneration, irrespective of the presence of fibrosis or Mallory bodies; (2) appropriate exclusion of other liver diseases such as alcoholic liver disease, viral hepatitis, drug-induced liver disease, primary biliary cirrhosis, biliary obstruction, autoimmune hepatitis, hemochromatosis, Wilson's disease and oc-1-antitrypsin (AAT) deficiency associated liver disease.

None of the patients had consumed alcohol in the previous six months, nor was there any history of surgery or ingestion of drugs known to produce hepatic steatosis during that time. Patients were diagnosed as diabetes mellitus if there was a documented use of oral hypoglycemic medication or insulin or fasting glucose greater than 140 mg/dl on two occasions.

Age, gender, height, weight, calculation of ideal body weight, reason for referral, symptoms at the time of presentation, other medical problems, and medications were all documented. All patients had a complete history and physical examination findings. Presence and absence of hepatomegaly was assessed clinically and by ultrasonography. Individuals with a body mass index (BMI) greater than 30 were recognized as having excessive body fat. In all cases, liver biopsies were performed as part of the evaluation of abnormal liver biochemistries.

This study was approved by the Ethics Group of Ankara University and all patients gave informed consent for participation.

Laboratory evaluation

Laboratory studies were obtained at the time of referral and included serum liver tests (alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total bilirubin, albumin and total protein levels); prothrombin time; hepatitis B and C serology (hepatitis B surface antigen, antibody to hepatitis B surface antigen, antibody to hepatitis B core antigen, antibody to hepatitis C virus, hepatitis C RNA polymerase chain reaction); autoimmune serology (antimitochondrial antibody-AMA, antinuclear antibody-ANA, anti smooth muscle antibody, anti liver/kidney microsomal antibody); studies of iron metabolism (fasting serum iron level, serum iron binding capacity (SIBC), transferrin saturation and ferritin level); studies of Cu metabolism (ceruloplasmin, copper excretion in 24-hour urine); and AAT levels. Serum urea nitrogen, creatinine, glucose, cholesterol, and triglyceride levels were also obtained. All laboratory analyses were performed by standard clinical laboratories.

Liver histology

A liver biopsy was performed in all patients and stained with hematoxylin-eosin and Masson's trichrome. All liver biopsies were evaluated by a single experienced hepatopathologist under coded identification without knowledge of the patients' clinical and biochemical findings. The level of fatty infiltration was assessed and graded on a scale of 1 to 3:l=mild (10-33% of hepatocytes affec-

ted); 2=moderate (34-67% of hepatocytes affected); 3=severe (>67% of hepatocytes affected). The degree of hepatic inflammation was graded on a scale of 1 to 3 (mild, moderate, severe). Presence or absence of Mallory bodies was recorded in all liver biopsies. The degree of fibrosis was assessed using a 5-grade scale; 0=none, normal connective tissue; l=mild, foci of pericellular fibrosis in zone 3; 2=moderate, perivenular or pericellular fibrosis confined to zone 3 and 2 regions, with or without portal/periportal fibrosis; 3=severe bridging or septal fibrosis; 4=cirrhosis. Hepatic iron was assessed by the grade of Perls' Prussian blue staining and hepatic copper was assessed by rhodamine stain.

Statistical analysis

Comparison of clinical and biochemical features between groups was done across levels of fibrosis and steatosis. Results are expressed as median (range), or number (proportion) of patients with a condition. Univariate analysis was performed using the nonparametric Kruskal-Wallis and Mann-Whitney tests to assess significance of differences of continuous variables among and between groups, respectively. The X^2 test was used for comparison of frequency data. The independent effect of significant variables ($p<0.05$) on fibrosis and steatosis was assessed using multiple regression analysis controlled for the effect of gender, with both the backward and forward stepwise selection procedures.

RESULTS

Patients' Demographics and Laboratory Evaluation

The main clinical and laboratory data of 53 patients with NASH are summarized in Table 1. The mean age was 41.5 ±1.1 (range 28-58) years; 17 (32%) patients were female. Six patients (11%) had diabetes mellitus (DM); 40 patients (75%) had hyperlipidemia; 6 (11%) patients had hypertension; 1 patient had history of alcohol use; 3 patients had hypothyroidism; and 3 patients had hyperthyroidism. The median body mass index (BMI) was 28 ± 0.5 with a wide range of 18 to 35. Obesity, as defined by a BMI greater than 30, was found in 30 (16 men, 14 female) patients (56%). Nine of 53 patients (17%) had right upper quadrant pain and the remaining 44 of 53 (83%) patients had no symptoms. Those patients were referred to our clinic because of elevated liver enzyme or ultrasound finding consistent with steatosis. None of the patients had ascites detected clinically or by ultrasound, or obvious peripheral edema.

Table 1. Demographic features of the patients

Parameter (normal value)	Mean ± SD	Range
BMI	28±0,5	18-35
Hb (13,2-17,3)	14.9±1.3	10.5-17
Htc (39-49)	43.4±3.9	30.4-49.5
ALT (0-40 U/L)	81.1±6.2	27-221
AST (0-37 U/L)	45.1±3.1	14-113
ALP (37-147 U/L)	85.5±4.7	38-220
GGT (0-57 U/L)	55.8±4.8	17-151
Fe (40-130 ng/dl)	108.1±5.4	33-190
SIBC (150-560 ng/dl)	400.2±13.4	179-652
TS (20 55 %)	27.2±1.5	10-63
Ferritin (28-365 ng/ml)	136.8±13.6	3.3-514
Total cholesterol (123-200 mg/dl)	201.5±5.5	91-316
Triglyceride (60-165 mg/dl)	203.1±12.8	46-393
Ceruloplasmin (0.2-0.6 g/dl)	0.28±0.01	0.16-0.5
AAT (1.4-3.2 g/dl)	1.3±0.03	0.7-1.8
U Copper (0-100 p.g/24 hour)	59.1±4.4	14-160

BMI: body mass index; Hb: hemoglobin; Htc: hematocrit; ALT: alanine aminotransferase; AST: aspartate aminotransferase; ALP: alkaline phosphatase; GGT: gamma glutamyl transferase; Fe: serum iron level; SIBC: serum iron binding capacity; TS: transferrin saturation; AAT: alpha-1 antitrypsin; U copper: 24-hour urinary copper

Five patients (9%; 3 female and 2 male) had decreased serum Hb concentration. The mean ALT/AST ratio was 1.8 in the total group of patients. An elevated serum ALT level was found in 47 patients (88%), AST in 26 (49%), gamma glutamyl transferase (GGT) in 23 (43%) and alkaline phosphatase (ALP) in 3 (5%) patients. Fourteen patients (26%; 11 male, 3 female) had elevated serum iron level, I patient had decreased serum iron level and 38 (71%) patients had normal serum iron level. An elevated SIBC was found in 2 (3.7 %; 1 male, 1 female) patients. Transferrin saturation was low in II (21%; 5 male, 6 female) patients and high in 2 (3.7%; 2 male) and normal in 30 patients. Serum ferritin level was high in 1 (male) patient, low in 3 (1 male, 2 female), and normal in 49 (92%) patients. One male patient had both elevated transferrin saturation and elevated serum iron concentration. Serum AAT level was normal in 30 patients and slightly low in 23 (43%; 18 male, 5 female). Serum ceruloplasmin level was normal in 49 (92%) and slightly low in 4 (7.5%; 2 male, 2 female) patients. Slightly elevated 24-hour urinary copper amount was found in 6 (11%) patients.

Serum viral markers (HBV, HCV), and ANA, anti ds-DNA and AMA were negative in all patients.

Liver biopsy findings

Liver biopsy findings are summarized in Table 2. NASH was grade I in 25 (47%) patients, grade II

in 20 (38%) and grade III in 8 (15%) patients. All patients had hepatic steatosis to different degrees and parenchymal inflammation of varying severity. Steatosis was found in all parenchymal zones as macro vesicular type in most patients. Lobular inflammation was found in all biopsy specimens and it was mild or moderate in 45 out of 53 (85%) patients and consisted of a mixed polymorphonuclear leukocyte and lymphocyte infiltration. No portal inflammation was found in 10 of 53 (19%) patients. Most patients (70%) had mild portal inflammation. Hepatic inflammation was mild or moderate in most patients. In 14 of 53 (26%) patients, steatosis and parenchymal inflammation were present without fibrosis. Most patients (59%) had

Table 2. Histological findings in liver biopsy

Steatosis (grade)	**No (%)**
1+	17 (32)
2+	16 (30)
3+	20 (38)
Ballooning (grade)	
1+	45 (85)
2+	8(15)
Lobular inflammation (grade)	
1+	25 (47)
2+	20 (38)
3+	8(15)
Portal inflammation (grade)	
0	10 (19)
1+	37 (70)
2+	6(11)
3+	None
Fibrosis (grade)	
0	14 (26)
1+	31 (59)
2+	7(13)
3+	1(2)
NASH (grade)	
I	25 (47.2)
II	20 (37.7)
III	8(15.1)
Iron	None
Copper	None

mild fibrosis. Perivenular or pericellular fibrosis confined to zone 3 and 2 regions, with or without portal/periportal fibrosis, was found in 7 of 53 (13) patients. None of our patients had bridging or septal fibrosis or cirrhosis. There was no staining of hepatic iron or copper in any patient.

Factors associated with the degree of NASH and fibrosis

There was no significant correlation between the degree of NASH and age, obesity, triglyceride, total cholesterol, ALT, AST, ALP, GGT and DM. There was significant correlation between serum total cholesterol level and the grade of fibrosis ($p= 0.03$). There was no significant correlation between the grade of fibrosis and age, obesity, triglyceride, total cholesterol, ALT, AST, ALP, GGT and DM.

DISCUSSION

The pathogenesis of NASH is poorly understood. Lipid peroxidation is probably the most important pathogenic mechanism in NASH, but other factors also likely contribute to the development of NASH either by enhancing lipid peroxidation or by directly stimulating fibrogenesis and the inflammatory response characteristic of NASH (2). There is controversial evidence that hepatic iron accumulation may play a role in the pathogenesis of NASH. Bacon et al. documented abnormal ferritin and/or transferrin saturation in 58% of their patients with NASH, some of whom had slightly elevated hepatic iron concentration (3). Angulo et al. assessed the independent predictors of liver fibrosis in patients with NASH and found that increased transferrin saturation correlated positively with the severity of fibrosis in univariant analysis. None of the patients in their study had increased hepatic iron concentration (9). Younossi et al. documented that increased iron was not seen in those with higher grades of fibrosis or other pathological features associated with the aggressive form of NASH. In most patients with NASH, significant iron accumulation is not seen. Additionally iron is not associated with poor clinical and pathological outcomes (5). Matteoni et al. showed that hepatic iron accumulation level was higher in men than in women with nonalcoholic fatty liver disease and showed no relationship between hepatic iron accumulation and aggressive histologic or clinical outcome in these patients (4). In our study, we showed no correlation between hepatic iron accumulation and steatohepatitis. Although we did not measure hepatic iron concentration in liver biopsy specimen, stainable hepatic iron accumulation is an important indicator for abnormal hepatic iron accumulation, and none of our patients with steatohepatitis had stainable iron accumulation in their liver biopsy specimen. Increased serum iron level was found in 14 of 51 (26%) patients, elevated transferrin saturation in 2 of 51 (3.7%), and high serum ferritin level in 1 of 51 (0.019%) patients. Abnormality in iron metabolism did not reflect the accumulation of iron in the liver. We found that abnormality in iron metabolism was not an

important factor in the development of steatohepatitis in our patient population.

George et al. reported that 31% of patients with NASH were either homozygous or heterozygous for the hemochromatosis mutation Cys 282 Tyr. Increased hepatic iron had the greatest association with the severity of fibrosis. The Cys 282 Tyr mutation is responsible for most of the mild iron overload found in NASH and thus has a significant association with hepatic damage in these patients (12). The prevalence of the hemochromatosis gene mutations associated with hereditary hemochromatosis is increased among North American subjects with NASH (13). It was not clear whether hepatic steatosis and inflammation led to increased serum ferritin and serum iron levels, iron accumulation was caused by heterozygosity for genetic hemochromatosis, or whether a mild increase in hepatic iron contributed to the liver injury in NASH. Iron could potentially play a supporting role in the lipid peroxidation and fibrogenesis central to the development and progression of NASH (2). Although we did not investigate hemochromatosis gene mutation, only one male patient in our study had both elevated serum transferrin saturation and elevated serum iron level. Nobody has investigated the incidence of hemochromatosis gene mutation in the Turkish population. Since only 1 of 51 patients had abnormality in serum iron concentration showing the possibility of hemochromatosis, genetic hemochromatosis was not an important epidemiologic factor in the development of steatohepatitis in our patient population.

Copper is an essential trace element in animals and man and both deficiency and excess may lead to disease (14). In the presence of available cellular reductants, copper in low molecular weight forms may play a catalytic role in the initiation of free radical reactions. The resulting oxyradicals have the potential to damage cellular lipids, nucleic acids, proteins and carbohydrates, resulting in wide-ranging impairment in cellular function and integrity (8). The liver and specifically the hepatocytes play a pivotal role in the metabolism of copper. The liver damage associated with Wilson disease may take many forms, from asymptomatic to chronic active hepatitis, cirrhosis, and even fulminant hepatitis with massive necrosis. In Wilson disease serum ceruloplasmin level is lower than 20mg/dl; serum copper level is lower than 100ug/dl; 24-hour urinary copper is higher than 100ug; post-penicillamine 24-hour urinary copper is higher than 1600 ug; and hepatic copper concentration is higher than 250ug/g dry wt. Microscopic examination of the liver biopsy specimen in the early stages of Wilson disease reveals microvesicular fatty changes and glycogen nuclei. Steatosis progresses to fibrosis and cirrhosis in untreated patients (15). Because none of our patients had any clinical sings of Wilson's disease, we did not measure copper content of liver biopsy specimen nor post-penicillamine 24-hour urinary copper content. We did not find significant abnormality in copper metabolism showing the possibility of Wilson disease in our patients. None of the patients had stainable copper in liver biopsy. Only one patient (male, 44 years old) had low serum ceruloplasmin level and high 24-hour urinary copper level (19 mg/dl and 144 ug, respectively), and he had no clinical findings of Wilson disease. This patient's NASH score was I and he had elevated serum ALT, AST and GGT level (74, 57, and 76 U/L, respectively). To our knowledge, this is the first study that investigates the relationship between the abnormality in copper metabolism and the pathogenesis of steatohepatitis. We found that copper metabolism is not an important factor in the development of steatohepatitis.

Obesity (especially central) (2,16-19), diabetes mellitus (2,17,18), insulin resistance (16,20), elevated serum leptin concentration (16,21), and hyperlipidemia (2,16,18) are the conditions most often reported in association with NASH. Some authors reported NASH to be predominant in females (60-83%) (2), and others reported predominance in males (16). By eliminating individuals' moderate-to-high alcohol consumption, hepatitis B or C virus, elevated transferrin saturation and a history of diabetes mellitus, Ruhi et al. used the elevated ALT as a surrogate for non-alcoholic fatty liver disease (17). They found that 2.8% of the population (5,724 participants) had an elevated ALT. Elevated ALT was associated with younger age, male sex, Mexican-American ethnicity, impaired glucose metabolism, insulin resistance, obesity, and higher leptin, triglyceride and C-peptide. The most common associated pathologic condition in our patients was hyperlipidemia (75.4%) and obesity defined by BMI as greater than 30 (56.7 %), and most of our patients were male (77.9%). As the first aim in this study was not to identify the epidemiologic features of patients with NASH, our study was not population based; most of our pati-

ents were referred to our clinic because of asymptomatic elevation of liver enzyme. Therefore, our findings do not reflect reliable epidemiologic features of patients with NASH in the Turkish population. Population based, multi-regional and larger population based studies are needed for this purpose.

Chitturi et al. identified female sex, diabetes mellitus and more severe hepatic inflammation as independent predictors of hepatic fibrosis, but not hemochromatosis gene mutations, serum ferritin, transferrin saturation or hepatic iron staining (10). Angulo et al. reported that body mass index, older age, obesity, diabetes mellitus, and a serum AST/ALT ratio greater than 1 were significant predictors of severe liver fibrosis (9). Serum leptin in NASH correlates with hepatic steatosis but not fibrosis (21). Our findings were not compatible with other findings. We did not find any correlation between the degree of NASH score and age, obesity, triglyceride, total cholesterol, ALT, AST, ALP, GGT, or DM. The only significant correlation determined was between serum total cholesterol level and the grade of fibrosis. Our study population was very small compared to that of Angulo et al. Predictors of the grade of NASH and that of fibrosis may be variable in different regions. The predominant cause of NASH, differences between genetic features of races, food consumption and other diseases associated with NASH may be important predictors in the advancement of fibrosis and NASH score.

In summary, we did not find any clinically important abnormality in the iron and copper metabolism or stainable iron and copper accumulation on the liver biopsy specimen in the 51 patients with NASH. The accumulation of iron and copper in the liver is not important in the etiopathogenesis of NASH.

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