

Causes of Thickening of Carotid Intima-Media Thickness in Patients With Alcoholic Liver Disease

A Prospective Observational Study

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Abstract: Several studies have reported the relationship between alcoholic liver disease (ALD) and carotid intima-media thickness (CIMT). Few studies, however, have investigated the causes of CIMT thickening in patients with ALD. The authors explored the causes of CIMT thickening in patients with ALD.

The authors enrolled 152 patients who were stratified into groups: nonthickening CIMT with ALD (group A); thickening CIMT with ALD (group B); nonthickening CIMT without ALD (group C); and thickening CIMT without ALD (group D).

The CIMT was significantly different between patients with and without ALD ($\chi^2=3.875$, $P=0.049$). The patients in groups A, B, and C were significantly younger than group D ($P=0.001$, 0.036 , and 0.001 , respectively). The body mass indexes (BMI) in groups A and B were significantly higher than in group C ($P=0.000$ and 0.007 , respectively). The blood glucose levels in groups B and D were significantly higher than in group C ($P=0.016$ and 0.018 , respectively). The blood uric acid levels in group B were significantly higher than in groups A, C, and D ($P=0.009$, 0.000 , and 0.003 , respectively). The blood uric acid in group A was significantly higher than in group C ($P=0.002$). The serum total cholesterol (TC) levels of patients in group B were significantly higher than in groups A and C ($P=0.027$ and 0.000 , respectively) and the serum TC level in group A was significantly higher than in group C ($P=0.048$). The serum triglyceride (TG) levels in groups A and B were significantly higher than in group C ($P=0.027$ and 0.000 , respectively). The serum of very low-density lipoprotein (VLDL) levels in group B were significantly higher than in group C ($P=0.000$). Although a comparison of the low-density lipoprotein (LDL) and high-density lipoprotein (HDL) serum levels among the 4 groups indicated no changes. The serum LDL levels in group B were significantly higher than in group A ($P=0.008$). No significant differences were observed among the groups with respect to serum homocysteine, C-reactive protein (CRP), interleukin-6 (IL-6), malondialdehyde (MDA), superoxide dismutase (SOD), soluble OX40 ligand (sOX40L), or heat shock protein (HSP) 60 or 70.

Alcoholic liver disease may result in CIMT thickening. Carotid intima-media thickness is associated with age and metabolic factors in patients with ALD. In addition, ALD might promote the premature occurrence of CIMT thickening. The thickening of carotid artery intima thickness, however, is not associated with cytokine profiles, oxidative balance, or immune responses in patients with ALD.

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Abbreviations: AFLD = alcoholic fatty liver disease, ALD = alcoholic liver disease, BMI = body mass index, CIMT = carotid intima-media thickness, CRP = C-reactive protein, ELISA = enzyme-linked immuno sorbent assay, Glu = glucose, Hcy = homocysteine, HD = hemodialysis, HDL = high-density lipoprotein, HSP = heat shock protein, IL-6 = interleukin-6, LDL = low-density lipoprotein, MDA = malondialdehyde, MetS = metabolic syndrome, NAFLD = nonalcoholic fatty liver disease, NASH = nonalcoholic steatohepatitis, SOD = superoxide dismutase, sOX40L = soluble OX40 ligand, TC = total cholesterol, TG = triglyceride, TNF- α = tumor necrosis factor alpha, UA = uric acid, VLDL = very low-density lipoprotein.

INTRODUCTION

Epidemiological studies have identified alcohol consumption as a factor that may either positively or negatively influence many diseases, including cardiovascular disease, certain cancers, and dementia. With the improvement in living standards, especially increases in drinking and daily alcohol intake levels, the incidence of nonalcoholic fatty liver disease (NAFLD), and alcoholic liver disease (ALD) continues to rise in China. Alcohol consumption is positively associated with carotid intima-media thickness (CIMT) in healthy men aged 30 to 70 years. In women, this correlation is not significant.¹ Non-alcoholic fatty liver disease has been associated with early alterations of the cardiovascular system, independent of established cardiovascular risk factors, and metabolic syndromes (MetS).² Immune and inflammatory pathways play a major role in the pathogenesis of NAFLD.³ Numerous studies have found that CIMT is associated with NAFLD.⁴⁻⁶ Recent literature has also reported that increased CIMT is associated with AFLD.^{5,6} Moreover, ageing, homocysteine (Hcy) levels, inflammation, lipid peroxidation, and immune response-related markers are associated with atherosclerosis. The mechanism of CIMT thickening in patients with alcoholic liver disease, however, remains controversial. Therefore, we investigated the relationships between metabolic-associated factors (age, BMI, blood lipid, blood glucose, blood uric acid, and Hcy), cytokine profile, oxidative balance, immune response, and CIMT thickening in patients with ALD.

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MATERIALS AND METHODS

Ethics Statement

Written informed consent was obtained from all patients before their participation. The protocol was approved by the clinical research ethics committee of Taishan Hospital in Shandong Province.

Study Population

From January 2012 to December 2013, we recruited 152 participants. Among these, 99 participants met the criteria for ALD established by the Fatty Liver and Alcoholic Liver Disease Group, Hepatology Branch, Chinese Medical Association.⁷ These patients also met the criteria for a diagnosis of chronic alcohol ingestion (daily ethanol intake higher than 40 g in men and 20 g in women for a period longer than 5 years).⁸ The remaining 53 participants did not have ALD. Participants were included based on inpatient and general health examinations. Based on ultrasonography results, a CIMT that was higher than or equal to 0.09 cm was considered as CIMT thickening. A CIMT less than 0.09 cm was considered normal. All patients were divided into 4 groups as follows: 61 patients with non-thickened CIMT with ALD (group A; 57 men, 4 women); 38 patients with thickened CIMT with ALD (group B; 35 men, 3 women); 41 patients with nonthickened CIMT without ALD (group C; 39 men, 2 women); and 12 patients with thickened CIMT without ALD (group D; 11 men, 1 woman). A cross-sectional study was conducted to determine the impact factors of CIMT in patients with ALD through the detection of serum levels of blood lipids, blood glucose, blood uric acid, Hcy, C-reactive protein (CRP), interleukin (IL)-6, tumor necrosis factor (TNF) α , malondialdehyde (MDA), superoxide dismutase (SOD), soluble OX40 ligand (sOX40L), heat shock protein (HSP) 60, and HSP70. Certain cases were excluded according to the exclusion criteria.⁹

Experimental Apparatus

A color ultrasound system (GEV7 and LOG7, GE), an automatic biochemical analyzer (7080, Japan), and a standard plate reader (ANTHOS2010, Austria) were used for this study. For a detailed description of the reagents mentioned below, please refer to the comments in our previously published manuscript.

Reagents

The following reagents were used: Hcy kit (provided by Michel Company, Sichuan, China); CRP kit (provided by Beijing JiuQiang Company); TNF α kit, MDA kit, SOD kit, sOX40L kit, HSP60 kit, and HSP70 kit (provided by Shanghai Enzyme-Linked Immune Co. LTD; all kits were manufactured by R&D companies).

Ultrasonic Investigation

Carotid intima-media thickness detection was performed by an ultrasonologist. The average CIMT was calculated from 3 separate values, which were obtained from a measurement of the vertical distance from the inner surface of the inner membrane to the external surface of the tunica media 1 cm proximal to the common carotid artery bifurcation in the left and right common carotid arteries.

Laboratory Tests

Venous blood was obtained from just above the elbow after all patients had fasted overnight for at least 10 hours. Serum was collected by centrifugation at 3000 rpm/min for 10 minutes then

preserved at -70°C . Blood lipids, blood glucose, and blood uric acid were detected via an automatic biochemistry analyzer. Serum CRP levels were detected by immunoturbidimetry and serum Hcy levels were detected using the velocity method. Serum levels of IL-6, TNF α , MDA, SOD, sOX40L, HSP60, and HSP70 were measured by ELISA. All test items were detected according to the manufacturer's instructions.

Statistical Analyses

Before the statistical analysis, the normal distribution and homogeneity of variances were assessed using the Kolmogorov test. Categorical variables were expressed as sample sizes (number of cases) and percentages (%) and quantitative variables were expressed as the mean \pm standard error (SEM) ($x \pm s$). The SPSS19.0 statistical package (SPSS Inc., Chicago, IL) was used for all statistical analyses. Categorical variables were compared using chi-square (χ^2) test. A one-way ANOVA was used to analyze multiple sample means. For multiple posthoc comparisons between groups, variables with normal distributions were analyzed using the LSD test and variables without normal distributions were analyzed using Tamhane test. *P* values less than 0.05 were considered significant.

RESULTS

A comparison of CIMT between ALD and non-ALD showed significant differences ($\chi^2 = 3.875$, $P = 0.049$).

A comparison of age, BMI, Glu, UA, and Hcy among the 4 groups is presented in Table 1. The differences in age, BMI, Glu, and UA were statistically significant ($F = 5.822$, $P = 0.001$; $F = 7.200$, $P = 0.000$; $F = 3.030$, $P = 0.031$; $F = 9.912$, $P = 0.000$, respectively). The ages of the patients in groups A, B, and C were significantly lower than in group D ($P = 0.001$, 0.036, and 0.001, respectively). The BMI of the patients in groups A and B were significantly higher than in group C ($P = 0.000$ and 0.007, respectively). The Glu serum levels of the patients in groups B and D were significantly higher compared with group C ($P = 0.016$ and 0.018, respectively). The UA serum levels of the patients in group B were significantly higher than in groups A, C, and D ($P = 0.009$, 0.000, and 0.003, respectively); group A was significantly higher compared with group C ($P = 0.002$). A comparison of the Hcy serum levels among the 4 groups indicated negligible changes, although the serum Hcy levels in groups B and D were slightly increased compared with groups A and C.

The differences in blood lipid levels among the 4 groups are shown in Table 2. The serum total cholesterol (TC), triglyceride (TG), and very low-density lipoprotein (VLDL) levels were significant ($F = 9.378$, $P = 0.000$; $F = 6.868$, $P = 0.000$; and $F = 6.403$, $P = 0.000$, respectively). The serum TC levels of the patients in group B were significantly higher than in groups A and C ($P = 0.027$ and 0.000, respectively) and the serum TC level in group A was significantly higher than in group C ($P = 0.048$). The serum TG levels in groups A and B were significantly higher than in group C ($P = 0.027$ and 0.000, respectively). The serum VLDL levels in group B were significantly higher than in group C ($P = 0.000$). In addition, although a comparison of LDL and HDL serum levels among the 4 groups indicated negligible changes, the serum LDL levels in group B were significantly higher than in group A ($P = 0.008$).

No significant differences were observed in the serum levels of CRP, IL-6, and TNF α among the 4 groups (Table 3). Although the *P* value for the serum TNF α level was significant ($F = 3.953$, $P = 0.010$), it failed the posthoc multiple comparisons test.

TABLE 1. A Comparison of Age, BMI, Glu, UA, and Hcy Among 4 Groups (Mean ± Standard Deviation)

| Group | N | Age (y) | BMI (kg/m ²) | Glu (mmol/L) | UA (pg/mL) | Hcy (μmol/L) |
|-------|-------|----------------|---------------------------|---------------------------|--------------------------------|---------------|
| A | 61 | 45.15 ± 6.63* | 26.74 ± 2.88 [†] | 5.58 ± 0.73 | 331.44 ± 75.63 ^{¶,‡‡} | 38.08 ± 16.96 |
| B | 38 | 48.37 ± 7.69** | 26.37 ± 2.49 [‡] | 5.81 ± 0.84 [§] | 376.39 ± 97.34 | 40.38 ± 17.55 |
| C | 41 | 45.80 ± 5.53* | 23.94 ± 2.05 | 5.42 ± 0.54 | 278.65 ± 75.39 [#] | 36.12 ± 11.28 |
| D | 12 | 53.00 ± 6.06 | 24.90 ± 3.41 | 5.98 ± 0.73 | 293.42 ± 83.56 [#] | 37.39 ± 12.12 |
| F | 5.822 | 7.200 | 3.030 | 9.912 | 0.632 | |
| P | 0.001 | 0.000 | 0.031 | 0.000 | 0.596 | |

BMI, body mass index, Glu, glucose, Hcy, homocysteine, UA, uric acid. ††P = 0.003 (UA: group D versus group B).

*P = 0.001 (Age: groups A and C versus group D).

**P = 0.036 (Age: group B versus group D).

[†]P = 0.000 (BMI: group A versus group C).

[‡]P = 0.007 (BMI: group B versus group C).

[§]P = 0.016 (Glu: group B versus group C).

^{||}P = 0.018 (Glu: group D versus group C).

[¶]P = 0.009 (UA: group A versus group B).

[#]P = 0.000 (UA: group C versus group B).

^{‡‡}P = 0.002 (UA: group A versus group C).

As presented in Table 4, a comparison of the serum levels of MDA and SOD among the 4 groups demonstrated no significant differences.

As presented in Table 5, no significant differences were observed for serum levels of sOX40L, HSP60, and HSP70 among the 4 groups.

DISCUSSION

Numerous studies have demonstrated that patients with NAFLD have increased CIMT and flow-mediated dilation, which are early markers of atherosclerosis¹⁰ and are associated with an increased risk of cardiovascular disease.^{11–13} Hepatic steatosis plays an important role in the increased risk for cardiovascular disease and is independent of insulin resistance (IR). Another study indicated that high-sensitivity C-reactive protein (hs-CRP) may represent a useful biomarker for this condition,¹⁴ which suggests that NAFLD may play a crucial role in the pathophysiology of the atherosclerotic process. The relationship between alcohol consumption and the risk for

metabolic syndrome in the general population, however, has been controversial. Age influences the relationships between alcohol consumption and atherosclerotic risk factors and a significant association has been observed between alcohol intake and a lower risk for metabolic syndromes in young men but not elderly men.¹⁵ Thus far, the previous studies have demonstrated that alcohol may affect the atherosclerotic process that underlies cardiovascular and cerebrovascular disease and the putative mechanisms involved. In particular, alcohol may affect vascular and endothelial function, smooth muscle cells,¹⁶ and atherosclerotic progression primarily through blood pressure regulation and lipid metabolism¹⁷. Dietary habits might be associated with steatohepatitis, as certain habits directly modulate hepatic triglyceride accumulation and antioxidant activity and other habits indirectly affect insulin sensitivity and postprandial triglyceride metabolism.¹⁸ In addition, patients with NAFLD have higher BMI, weight, and waist circumference compared with healthy individuals. In addition, serum levels of liver enzymes, TG, LDL, BUN, and uric acid were higher in

TABLE 2. Comparison With Blood Lipid Between 4 Groups (mmol/L, Mean ± Standard Deviation)

| Group | N | Blood | | Lipid | | |
|-------|----|---------------------------|--------------------------|---------------------------|-------------|--------------------------|
| | | TC | TG | LDL | HDL | VLDL |
| A | 61 | 5.36 ± 0.94* [¶] | 2.68 ± 2.17 [†] | 2.89 ± 0.99 | 1.30 ± 0.13 | 1.21 ± 0.98 |
| B | 38 | 6.06 ± 1.25 | 2.84 ± 2.31 [†] | 3.43 ± 1.01 | 1.33 ± 0.11 | 1.22 ± 0.97 [§] |
| C | 41 | 4.89 ± 0.81** | 1.18 ± 0.55 | 3.03 ± 0.87 | 1.32 ± 0.28 | 0.54 ± 0.24 |
| D | 12 | 5.22 ± 0.81 | 1.89 ± 1.78 | 3.08 ± 0.97 | 1.32 ± 0.17 | 1.00 ± 0.88 |
| F | | 9.378 | 6.868 | 2.462 | 0.238 | 6.403 |
| P | | 0.000 | 0.000 | 0.065 | 0.087 | 0.000 |

HDL, high density lipoprotein; LDL, low-density lipoprotein; TC, total cholesterol; TG, triglyceride; VLDL, very low-density lipoprotein.

[§]P = 0.000 (VLDL: group B versus group C).

*P = 0.001 (TC: group A versus group B).

**P = 0.001 (TC: group C versus group B).

[†]P = 0.027 (TG: group A versus group C).

[‡]P = 0.000 (TG: group B versus group C).

^{||}P = 0.008 (LDL: group A versus group B).

[¶]P = 0.048 (TC: group A versus group C).

TABLE 3. Comparison of Serum CRP, IL-6, and TNF- α Among 4 Groups (ng/L, Mean \pm Standard Deviation)

| Group | N | CRP | IL-6 | TNF- α |
|-------|----|-----------------|-------------------|---------------------|
| A | 61 | 4.04 \pm 3.67 | 16.97 \pm 14.74 | 264.19 \pm 224.85 |
| B | 38 | 4.25 \pm 1.80 | 22.12 \pm 16.98 | 270.44 \pm 290.63 |
| C | 41 | 3.80 \pm 3.21 | 25.41 \pm 25.92 | 460.38 \pm 442.81 |
| D | 12 | 4.07 \pm 2.11 | 29.08 \pm 27.09 | 430.43 \pm 327.50 |
| F | | 0.145 | 2.173 | 3.953 |
| P | | 0.933 | 0.094 | 0.010 |

CRP, C-reactive protein; IL-6, interleukin-6; TNF- α , tumor necrosis factor- α .

patients with NAFLD.¹⁹ The results of the current study demonstrate that ALD may result in CIMT thickening, supporting Kim’s viewpoint.⁴ Meanwhile, age, BMI, Glu, UA, TC, TG, VLDL, and LDL are important risk factors for CIMT thickening in patients with ALD.

Many previous studies have reported associations among aging, Hcy levels, and atherosclerosis, but these associations have remained controversial. Studies have confirmed that Hcy may promote neuronal damage through multiple mechanisms, including microvascular damage that is mediated by an increase in IMT and a direct neurotoxic effect.²⁰ Advanced age affects Hcy levels and IMT is a useful marker of atherosclerosis in hemodialysis (HD) patients.²¹ Another study based on prepubertal children demonstrated that only overweight and obese children demonstrated associations with increased Hcy concentrations ($\geq 7.3 \mu\text{mol/L}$).²² Homocysteine, however, is not an independent factor in the genesis of atherosclerosis in HD patients.¹⁹ Homocysteine and age are not similarly related to the arterial augmentation index (AAI) and IMT in asymptomatic individuals.²³ In this study, the results indicate an obvious statistical difference for age but not serum Hcy levels among the 4 groups. The data suggest that carotid intimal thickening in patients with ALD is associated with age and this occurs prematurely in patients with ALD. Carotid intimal thickening, however, has no relationship with serum homocysteine levels. Inflammation is associated with both NAFLD and atherosclerosis. A study²⁴ showed that serum ferritin, inflammatory cytokines, and oxidative stress markers were significantly higher in nonalcoholic steatohepatitis (NASH) patients, which suggests that the accumulation of iron and fat in the liver, oxidative stress, and inflammatory cytokines are closely related. Furthermore, the levels of serum ferritin, MDA, IL-6, TNF α , and IL-8

TABLE 4. Compare on Serum MDA and SOD Among 4 Groups (Mean \pm Standard Deviation)

| Group | N | MDA (ng/L) | SOD (U/mL) |
|-------|-------|-------------------|--------------------|
| A | 61 | 6.80 \pm 6.23 | 65.08 \pm 62.15 |
| B | 38 | 7.71 \pm 8.16 | 76.27 \pm 67.99 |
| C | 41 | 10.87 \pm 10.87 | 94.87 \pm 109.49 |
| D | 12 | 8.73 \pm 8.95 | 98.03 \pm 97.42 |
| F | | 2.006 | 1.337 |
| P | 0.116 | 0.265 | |

MDA, malondialdehyde; SOD, superoxide dismutase.

TABLE 5. Compare on Serum sOX40L, HSP60, and HSP70 Among 4 Groups (ng/L, Mean \pm Standard Deviation)

| Group | N | sOX40L | HSP60 | HSP70 |
|-------|----|-------------------|-------------------|-------------------|
| A | 44 | 9.38 \pm 7.52 | 67.06 \pm 50.78 | 51.16 \pm 34.97 |
| B | 54 | 11.89 \pm 15.62 | 82.42 \pm 72.24 | 44.63 \pm 22.53 |
| C | 44 | 12.86 \pm 12.41 | 65.13 \pm 54.86 | 54.83 \pm 36.46 |
| D | 20 | 16.88 \pm 18.57 | 72.64 \pm 55.72 | 73.00 \pm 64.26 |
| F | | 1.546 | 0.721 | 1.948 |
| P | | 0.205 | 0.541 | 0.124 |

HSP60, heat shock protein; HSP70, heat shock protein 70; sOX40L, soluble OX40 ligand.

may represent indices of activity and progression of NASH, considering that insulin resistance and a systemic inflammatory response are very important during the induction of NAFLD, particularly in apparently healthy nonobese men. Conflicting results⁶ suggest that hsCRP measurement for the identification of a hepatic inflammatory response in patients with MetS alongwith NAFLD is limited because of its low sensitivity and specificity as observed after the identification of different degrees of hepatic inflammation. In addition, growing evidence indicates that inflammatory reactions play an important role in the pathogenesis of ALD. Our studies demonstrate that the increases in serum markers of inflammation are coupled with CIMT thickening in patients with ALD; however, the levels of these serum markers did not significantly differ between the groups. Therefore, serum markers of inflammation are not associated with carotid artery intimal thickening in patients with ALD.

The involvement of free-radical mechanisms in the pathogenesis of ALD has been demonstrated by the detection of lipid peroxidation in the liver and the serum of patients with alcoholism.^{25,26} In addition, experiments in alcohol-fed rodents have demonstrated a relationship between alcohol-induced oxidative stress and the development of liver pathology.²⁵ Recent studies²⁷ have indicated that oxidative stress is highly prevalent in children with NAFLD and is associated with an increased severity of steatohepatitis. In addition, elevated MDA levels may indicate a relationship between oxidative stress and NASH.²⁸ Other data indicate that the presence of an immune reaction is triggered by oxidative stress and can be an independent predictor of NAFLD progression to advanced fibrosis.²⁹ The attenuation of alcoholic hepatitis is associated with decreased oxidative stress.³⁰ During ALD, Kupffer cells are involved in the generation of protein adducts with both acetaldehyde- and ethanol-induced lipid peroxidation products.³¹ Serum MDA concentrations increase with the severity of ALD.³² Our research demonstrated increased CIMT in patients with ALD, enhanced lipid peroxidation, and increased antioxidants. These differences, however, were not significant. We considered that lipid peroxidation and antioxidants are not associated with intimal thickening in patients with ALD. This possibility requires further research.

The relationship between the immune response and CIMT in patients with ALD has not been well investigated. A previous study demonstrated that sOX40L is independently related to CIMT, which implies a possible relationship of sOX40L with atherosclerosis.³³ The positive relationship between sOX40L and CRP and the negative relationship with IL-10 suggests possible proinflammatory effects of sOX40L on the

pathogenesis of atherosclerosis. Heat shock proteins are powerful immunogens against the antigenic peptides they chaperone.³⁴ Furthermore, HSPs are overexpressed in a wide range of human cancers and have been implicated in tumor cell proliferation, differentiation, invasion, metastasis, death, and recognition by the immune system.³⁵ The enhanced biliary expression of HSP60 is a common feature of chronic biliary disease irrespective of etiology and is not specific to autoimmune diseases.³⁶ Increased HSP60 expression during chronic active hepatitis suggests that immune reactions to HSP60 may play a role in the immunopathogenesis and perpetuation of chronic inflammatory liver disease.³⁷ The current experiments demonstrated that CIMT thickening in patients with ALD is not accompanied by obvious changes in serum levels of sOx40L or HSP60, which indicates that the immune response-related markers are not associated with carotid artery intimal thickening in patients with ALD.

In conclusion, ALD may result in CIMT thickening. Carotid intima-media thickness is associated with age and metabolic-associated factors, that is, BMI, Glu, UA, TC, TG, VLDL, and LDL in patients with ALD. In addition, ALD might prompt the premature occurrence of CIMT thickening. Carotid intima-media thickness, however, was not associated with cytokine profiles, oxidative balance, or immune response. Furthermore, because the number of samples in this study was small, future studies with more patients are required to verify these results.

Our study has certain limitations. First, the study samples were small and biased by a disproportionate number of male patients. Second, the confounding factors, such as drugs, obesity, and other risk factors, were unknown. Third, this study did not distinguish the effects of the variety or quantity of drinking on CIMT in patients with ALD.

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