Changes in Tumor Necrosis Factor-α, Heat Shock Protein 70, Malondialdehyde, and Superoxide Dismutase in Patients With Different Severities of Alcoholic Fatty Liver Disease

A Prospective Observational Study

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Abstract: The relationships among inflammation, oxidative balance, and the severity of alcoholic fatty liver disease (AFLD) remain unknown. The aim of this study is to explore the relationships among tumor necrosis factor alpha (TNF-α), heat shock protein 70 (HSP70), malondialdehyde (MDA), superoxide dismutase (SOD), and the severity of AFLD.

From January 2012 to December 2013, 162 participants were enrolled in this study and divided into 4 groups: 44 cases of mild AFLD (group A), 55 cases of moderate-to-severe AFLD (group B), 44 cases of alcohol consumption without AFLD (group C), and 20 cases of no alcohol consumption without AFLD (group D). A cross-sectional study was conducted by detecting the serum levels of TNF-α, HSP70, MDA, and SOD by enzyme-linked immunosorbent assay.

The median serum levels of TNF-α and HSP70 among the 4 groups were statistically significant (P = 0.000 and 0.001, respectively). The median serum levels of TNF-α in groups A and B were significantly lower than in group C (P = 0.002 and 0.000, respectively), and the median serum level of TNF-α in group B was significantly lower than in group D (P = 0.023). In addition, the median serum level of HSP70 in group B was significantly lower than in groups A and C (P = 0.002 and 0.000, respectively), and the median serum level of HSP70 in group C was significantly higher than in group D (P = 0.044). However, the median serum level of MDA in group B was significantly lower than only group C (P = 0.008).

Chronic alcohol ingestion without AFLD may result in a significant increase in the circulation of certain inflammatory markers; the severity of AFLD is associated with circulating inflammatory markers, and moderate-to-severe AFLD may result in a more significant reduction of these markers. However, moderate-to-severe AFLD may also result in a significant downregulation of oxidative stress products.

INTRODUCTION

The quantity of alcohol consumed in China is enormous, and its consumption is related to multiple health problems. To this point, the clinical diagnostics for fatty liver disease commonly include abdominal ultrasonography (US), computerized tomography (CT), magnetic resonance imaging (MRI), and others. The differences between nonalcoholic steatohepatitis (NASH) and nonprogressive non-alcoholic fatty liver disease (NAFLD) were not apparent with any radiological modality (including US, CT, and MRI). Of the pathologic features important for establishing the diagnosis of NASH, only the severity of steatosis was reflected in these radiological modalities. However, because ultrasound is low cost, safe, and accessible, it is the imaging technique of choice for screening for fatty livers in clinical and population settings. The utility of US for noninvasive diagnosis and the estimation of hepatic steatosis has been demonstrated in a large prospective pediatric study. Ultrasound has a high accuracy in the diagnosis and grading of steatosis and fibrosis in hepatitis C virus (HCV) nonresponders. In addition, US allows for reliable and accurate detection of moderate-to-severe fatty liver disease compared with histology. Therefore, US is widely used in clinical settings, and alcohol-induced fatty liver disease is a common finding during abdominal ultrasounds at routine health check-ups. The liver is the organ that controls ethanol metabolism, and it is susceptible to the toxic effects of alcohol. The metabolism of ethanol generates a number of metabolites, including acetate, reactive oxygen species (ROS), and acetaldehyde, and produces epigenetic changes, which can induce inflammatory responses. Growing evidence illustrates that alcoholic liver disease (ALD) is associated with inflammatory responses, oxidative stress, and the immune response. However, the associations among inflammation, oxidative balance, and the severity of ALD are unknown. For this purpose, enzyme-linked immunosorbent assays (ELISAs) were used to examine serum levels of tumor necrosis factor alpha (TNF-α), heat shock protein 70 (HSP70), malondialdehyde (MDA), and superoxide dismutase (SOD) in order to investigate the associations among inflammation, oxidative balance, and the severity of AFLD.

Abbreviations: AFLD = alcoholic fatty liver disease, ALD = alcoholic liver disease, HSP = heat shock protein, MDA = malondialdehyde, NASH = nonalcoholic steatohepatitis, ROS = reactive oxygen species, SOD = superoxide dismutase, TNF-α = tumor necrosis factor alpha.

DOI: 10.1097/MD.000000000000643
MATERIALS AND METHODS

Ethics Statement
All participants were enrolled after obtaining informed consent. The protocol for this study was evaluated and approved by the Research Ethics Committee of Taishan Hospital of Shandong Province, China.

Study Population
From January 2012 to December 2013, 142 participants with chronic alcohol ingestion and 20 control participants were enrolled in this study. After abdominal ultrasonographic examination according to the criteria for AFLD established by the Fatty Liver and Alcoholic Liver Disease Group, Hepatology Branch, Chinese Medical Association,10 participants were divided into 4 groups. These groups included 44 cases of mild AFLD (group A: 41 males and 3 females with an average age of 46.58 ± 6.56 years); 54 cases of moderate-to-severe AFLD (group B: 50 males and 4 females with an average age of 46.33 ± 6.79 years); 44 cases of chronic alcohol consumption without AFLD (group C: 42 males and 2 females with an average age of 48.08 ± 6.67 years); and 20 cases of no chronic alcohol consumption without AFLD (group D: 18 males and 2 female with an average age of 44.76 ± 5.49 years). There were no significant differences among the groups with regard to average age (P > 0.05). A cross-sectional study was conducted to determine inflammatory, oxidative balance, and the severity of AFLD through the separate detection of serum levels of TNF-α, HSP70, MDA, and SOD.

Exclusion criteria for cases included the following factors: age < 25 years or > 70 years, smoking, fever, pregnancy, women attempting to become pregnant, lactating women, individuals who were HBsAg positive or hepatitis B virus–DNA positive, those infected with HCV as confirmed by serologic tests, and patients with autoimmune hepatitis, cholestatic hepatitis, liver cancer, hepatic encephalopathy, electrolyte and acid-base balance disorders, gastrointestinal bleeding, infection, hepatic decompensation, primary or/and secondary heart, head, endocrine, nervous, or hematological diseases, or mental disorders.

Experimental Apparatus
A color ultrasound system (GEV7 and LOG7; GE) and a standard plate reader (ANTHOS2010; Austria) were utilized for this study. For a detailed description of the reagents listed further, please refer to the comments in our previously published manuscript.

Reagents
The reagents used include a TNF-α kit, a HSP70 kit, a MDA kit, and a SOD kit (provided by Shanghai Enzyme-Linked Immune Co. Ltd, Shanghai, China; all kits were manufactured by R&D companies, USA).

Laboratory Tests
Venous blood was drawn from just above the elbow after all patients had fasted overnight for at least 10 hours. Serum was collected by centrifugation at 3000 rpm for 10 minutes and then preserved at −70°C. Serum levels of TNF-α, HSP70, MDA, and SOD were measured by ELISA. All test items were detected according to the manufacturer’s instructions.

Ultrasonic Investigation
After an overnight fast, abdominal ultrasound examinations were performed by an experienced ultrasonographer with a 3.5 to 5 MHz convex probe and a high-resolution B-mode US scanner. The degrees of steatosis at US were divided into 3 grades11: mild, moderate, and severe steatosis.

Statistical Analysis
Quantitative variables are expressed as the medians with ranges. All data analyses were conducted using the SPSS19.0 statistical package (SPSS Inc., Chicago, IL). A Kruskal–Wallis analysis of variance was used for comparisons among the 4 groups, and a Mann–Whitney U test was used for comparisons between groups. P values < 0.05 were considered statistically significant.

RESULTS
The change in the median serum levels of TNF-α and HSP70 is shown in Table 1. The median serum levels of TNF-α among the 4 groups were significantly different, H = 20.871, P = 0.000. The median serum levels of TNF-α in groups A and B were significantly lower than in group C, P = 0.002 and 0.000, respectively. The median serum level of TNF-α in group B was significantly lower than in group D, P = 0.023. In addition, significant differences were found in the median serum levels of HSP70 among the 4 groups, H = 17.699, P = 0.001. The median serum level of HSP70 in group B was significantly lower than in groups A and C, P = 0.002 and 0.000, respectively, and the median serum level of HSP70 in group C was significantly higher than in group D, P = 0.044.

The changes in the median serum levels of MDA and SOD among the 4 groups are shown in Table 2. The median serum levels of MDA and SOD in groups A and B were lower than in groups C and D; however, no significant differences were observed among the 4 groups. Comparisons between the groups showed that the median serum level of MDA in group B was significantly lower than in group C, P = 0.008.

DISCUSSION
Ethanol (alcohol) is commonly known to alter cytokine levels in the plasma, lung, liver, and brain.12 Growing evidence indicates that chronic alcohol ingestion complicated by ALD is characterized by the activation of inflammatory responses and generates inflammatory cytokines. Inflammatory reactions play an important role in the pathogenesis of ALD. Cytokines are multifunctional proteins that play a critical role in cellular function.

| TABLE 1. Change in the Serum Levels of TNF-α and HSP70 Among the 4 Groups (ng/L, Medians With Ranges) |
|-------------|--------|--------|
| Group       | n      | TNF-α  | HSP70   |
| A           | 44     | 193.55 (1.50–1337.00) | 43.90 (21.40–311.00) |
| B           | 54     | 170.00 (99.60–1742.00) | 36.75 (21.40–276.00) |
| C           | 44     | 265.50 (123.60–2078.20) | 48.40 (27.10–228.30) |
| D           | 20     | 207.65 (176.30–1676.00) | 36.65 (21.40–92.80) |
| H           | 20     | 20.871 | 17.699  |
| P           | 0.000  | 0.000  | 0.001   |

HSP70 = heat shock protein 70, TNF-α = tumor necrosis factor-α.

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communication and activation and impact a variety of tissues in a complex manner that regulates inflammation, cell death, cell proliferation and migration, as well as healing mechanisms. In addition, TNF-α is a cytokine involved in systemic inflammation during acute-phase reactions. A previous study found a significant increase in the spontaneous production of inter-leukin (IL)1β, IL6, IL12, and TNF-α in peripheral blood monocytes among individuals actively consuming ethanol. Conversely, another study found that acute alcohol exposure modestly inhibited TNF-α production. However, by day 6, ethanol consumption significantly upregulated TNF-α production in association with an increased generation of ROS. Accumulating evidence suggests that proinflammatory cytokines, such as TNF-α, might cause hepatocellular damage and that hepatoprotective cytokines, such as IL6, have protective effects on hepatocytes. In addition, an animal experiment in rats showed that TNF-α and IL6 serum levels may be used as predictive biomarkers for ALD progression. HSP70, as an intracellular polypeptide, can be exposed on plasma membranes and/or released into circulation, eliciting immune responses that may contribute to vascular damage. A significant increase in HSP70 serum levels is associated with an increasing degree of inflammation. Recent studies have identified circulating HSP as an important mediator in inflammation, and the effects of low-grade inflammation on the aging process are overwhelming. The serum concentration of HSP70 decreases with age in the normal population, illustrating that higher levels of HSP70 contribute to nonalcoholic steatohepatitis. Reduced paraoxonase-1 activity may contribute to NAFLD. In-vivo models of alcohol infusion induce lipid peroxidation products may also generate oxidative stress and significantly contributes to liver injury. ROS induction is involved in ethanol-enhanced TNF-α production by monocytes. Moreover, the role of immune responses triggered by oxidative stress was substantiated in the progression of NASH. In addition, oxidative stress is also a major contributing factor to the pathogenesis of ALD and NAFLD. In-vivo models of alcohol infusion induce lipid peroxidation products because of increased free radical formation and decreased levels of hepatic antioxidants such as GSH. Patients with NAFLD show enhanced oxidative stress, which may lead to nonalcoholic steatohepatitis. Reduced paraoxonase-1 activity and increased MDA levels could be considered biochemical markers for lipid peroxidation. Previous studies have shown that human NASH is often associated with the presence of circulating antibodies against protein adducted by lipid peroxidation products. The disturbed metabolism of superoxide due to the decreased activities of SOD and catalase seem to be important in the pathogenesis of NASH. Sakaguchi et al emphasized the important role of gut-derived bacterial toxins, the innate immune system, and oxidative stress in the common pathogenic mechanism of ALD and NASH progression. Oxidative stress plays an important role in the development of liver damage resulting from alcohol consumption. The data from Abdelmeged et al indicated that both intestinal and hepatic CYP2E1 induced by binge alcohol consumption appear to be critical in binge alcohol-mediated increases in nitrooxidative stress, gut leakage, endotoxemia, altered fat metabolism, and inflammation. Our study reached a different conclusion, namely that only the median serum level of MDA in moderate-to-severe

### TABLE 2. Change in the Serum Levels of MDA and SOD Among the 4 Groups (MDA/μg/L, SOD/μmol/L, Medians With Ranges)

| Group | n  | MDA       | SOD       |
|-------|----|-----------|-----------|
| A     | 44 | 4.44 (2.00–41.00) | 54.75 (9.90–310.70) |
| B     | 54 | 4.64 (2.00–47.00) | 38.80 (4.50–343.90) |
| C     | 44 | 6.21 (3.00–43.00) | 41.25 (16.40–532.40) |
| D     | 20 | 6.21 (1.90–39.00) | 40.20 (24.20–318.10) |
| H     | 7  | 7.569 | 3.175 |
| P     | 0.056 | 0.365 |

MDA = malondialdehyde, SOD = superoxide dismutase.

*P* = 0.008 (MDA: group B vs group C)
AFLD was significantly lower than in chronic alcohol ingestion without fatty liver disease, suggesting that moderate-to-severe AFLD may result in the downregulation of MDA. At present, the role of MDA downregulation is not entirely clear and requires further research.

CONCLUSIONS

Chronic alcohol ingestion without AFLD may result in a significant increase in the circulation of certain inflammatory markers (e.g., HSP70). The severity of AFLD is associated with the circulation of certain inflammatory markers (e.g., TNF-α and HSP70), and moderate-to-severe AFLD may result in a more significant reduction of circulating inflammatory markers. In addition, moderate-to-severe AFLD may also result in the significant downregulation of oxidative stress products (e.g., MDA).

Our study has certain limitations. First, the subject study sample was small and biased by a disproportionate number of male individuals. Second, our conclusions are based on a prospective observational study, and the individuals were selected by whether they had chronic alcohol ingestion or neither chronic alcohol ingestion nor AFLD, rather than a completely randomized selection of individuals.

ACKNOWLEDGMENTS

The authors would like to thank Mr Sun Yi-Sheng and Ms Wang Hong for their technical assistance. This article was re-edited by American Journal Experts.

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