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The relationship between endoplasmic reticulum stress and liver function, insulin resistance and vascular endothelial function in patients with non-alcoholic fatty liver disease

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Abstract. – OBJECTIVE: The aim of the study was to investigate the relationship between ER stress and liver function, insulin resistance and vascular endothelial function in patients with non-alcoholic fatty liver disease.

PATIENTS AND METHODS: A total of 95 patients with non-alcoholic fatty liver disease were selected. They were admitted to our hospital from November 2016 to January 2019. A total of 90 cases of obese patients without fatty liver were selected as control group during the same period. The levels of ER stress marker protein were compared between the two groups, and the relationship between ER stress and liver function, insulin resistance, and vascular endothelial function was analyzed.

RESULTS: The protein level of ER stress markers in the test group was significantly higher than that in the control group ($p<0.05$). The liver function index and insulin resistance level were significantly higher than those in the control group ($p<0.05$). The level of vascular endothelial function was significantly lower than that of the control group ($p<0.05$). Pearson correlation analysis showed that ER stress marker protein was positively correlated with liver function and insulin resistance ($p<0.05$), while ER marker protein was negatively correlated with vascular endothelial function ($p<0.05$).

CONCLUSIONS: Liver function and insulin resistance are closely related to ER stress in patients with non-alcoholic fatty liver disease. Insulin resistance is one of the factors inducing and aggravating endothelial dysfunction.

Key Words:

Non-alcoholic fatty liver disease, Endoplasmic reticulum stress, Liver function, Insulin resistance, Vascular endothelial function.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is a clinical pathological syndrome. It is characterized by excessive fat deposition in hepatocytes caused by alcohol and other definite liver damaging factors[1]. The diseases include simple fatty liver (SFL), non-alcoholic steatohepatitis (NASH), and related cirrhosis. With the globalization trend of obesity and its related metabolic syndrome[2], non-alcoholic fatty liver disease has become an important part of chronic liver disease in developed countries, such as Europe, America, and rich regions of China. The prevalence of NAFLD in general adults is relatively high, most of which are NASH. The incidence of cirrhosis in NASH is greatly increasing within the recent 10 years. Endoplasmic reticulum[3] is an important site for post-synthesis modification, folding and operation of proteins. It is also an important Ca^{2+} reservoir in cells. Internal environment disorders can lead to accumulation of unfolded proteins in the ER, protein folding, transport dysregulation, and intracellular Ca^{2+} balance disorders, leading to endoplasmic reticulum stress (ERS). Endoplasmic reticulum stress is closely related with hepatitis B, non-alcoholic fatty liver disease (NAFLD), Alzheimer's disease and diabetes mellitus[4].

The etiology of NAFLD is not yet clear. It is generally believed that the main causes of NAFLD are genetic susceptibility[5], insulin resistance (IR)[6,7], oxygen stress[8], apoptosis[9], and injury caused by adipocytokine[10]. It was speculated that IR is the main link of the occurrence and development of NAFLD. IR refers to the insensitivity of target tissues to insulin. It can reduce the biological effects of insulin on glucose uptake and utilization. The compensatory excessive secretion of insulin by islet β cells is mainly characterized by hyperinsulinemia with metabolic stress syndrome. Ibrahim et al[11] have shown that fatty liver can reduce liver degrada-

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tion of insulin and insulin sensitivity. Moreover, we hypothesized that ER stress in patients with non-alcoholic fatty liver disease could have an impact on IR, liver function and vascular endothelial function.

However, besides IR, NAFLD is clinically observed to be accompanied by cardiovascular disease (CVD)[12]. Nowadays, many scholars believe that NAFLD is the manifestation of multiple sclerosis (MS) in the liver system. The emergence of NAFLD may not only be a marker of CVD, but also an early and intermediate state of CVD[13]. Endothelial dysfunction is an early change of atherosclerosis. Therefore, this study discussed the relationship between ER stress and liver function, insulin resistance and vascular endothelial function in patients with non-alcoholic fatty liver disease.

Patients and Methods

General Information

A total of 95 patients with non-alcoholic fatty liver disease were selected as the test group. They were admitted to our hospital from November 2016 to January 2019. There were 70 males and 25 females. Another 90 patients who came to our hospital for obesity but no fatty liver during the same period was selected, including 67 males and 23 females. This study was designed to investigate the relationship between ERS, IR, liver function and vascular endothelial function in the pathogenesis of NAFLD in two groups.

Inclusion and Exclusion Criteria

The inclusion criteria were as follows: patients who met the clinical diagnostic criteria for non-alcoholic fatty liver disease after CT and liver function examination[14], including patients who had no history of drinking or the alcohol content of alcohol less than 40 g per week, and the liver histological changes met the pathological diagnostic criteria of fatty liver disease; patients who were confirmed as obese by physical examination but had no fatty liver. The exclusion criteria were as follows: hard drinkers; patients with autoimmune hepatitis; patients with specific diseases such as viral hepatitis and total parenteral nutrition that can cause fatty liver; patients with drug-induced liver injury; patients who were not willing to participate in the experiment. All subjects and their families agreed to participate in the experiment and signed the informed consent. This trial was in accordance with the Helsinki Declaration, and has been approved by the Ethics Committee of the hospital.

Observation Indicators

The following indicators were observed: height, body mass, waist circumference (meridian at the midpoint of the line between the lower rib margin and the anterior superior iliac spine), hip circumference (meridian at the level of femoral trochanter) and BMI. Venous blood was taken in the next morning after fasting for 10 hours to measure the liver function. The liver function indicators, including triglyceride (TG), alanine aminotransferase (ALT) and aspartate aminotransferase (AST), were measured. Fasting blood glucose (FBG), fasting insulin (FNS), and insulin resistance (IR) were calculated by steady-state model method. The endothelium-dependent vasodilation (FMD) was detected by ultrasound, and the expression of cell markers GRP78, XBP1, CHOP and caspase-12 was detected by Western blot.

Test Methods and Materials

Western blot[15] was used to detect the hepatocyte marker protein expression in each group. Approximately 50 mg of hepatocytes was taken from each group, and 1 mL lysate was added, then they ground in a homogenizer, and rapidly homogenized at 4°C. Then, the homogenate was removed to a 1.0 mL pipette. After centrifugation at 12000 × g, at 4°C for 2 min, the supernatant was collected as the protein solution to be analyzed. The protein concentration was determined by Coomassie brilliant blue colorimetry. Each sample was loaded with 50 L. After separation by electrophoresis, the protein was transferred to a polyvinylidene difluoride (PVDF) membrane. 3% skimmed milk powder and 3% BAS sealing solution were sealed. Rabbit anti-mouse GRP78 polyclonal antibody (1:500), rabbit anti-mouse XBP-1 polyclonal antibody (1:500), rabbit anti-mouse CHOP polyclonal antibody (1:500), rabbit anti-mouse caspase, 12 polyclonal antibody (1:500) and rabbit anti-mouse GAPDH antibody (1:500) were added respectively. The second antibody (HRP-labeled goat anti-rabbit IgG, dilution ratio 1:500) was added and rinsed with Tris-Buffered Saline and Tween (TBST) three times. Western blot hypersensitive luminescent solution was added to detect the expression of target protein by chemiluminescence under exposure

of FUJInLM LAS-3000 imager. Rabbit anti-rat GRP78 polyclonal antibody and rabbit anti-rat caspase-12 polyclonal antibody were purchased from Sigma-Aldrich (St. Louis, MO, USA); rabbit anti-mouse XBPl polyclonal antibody, rabbit anti-mouse CHOP polyclonal antibody and rabbit anti-mouse GAPDH antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, , CA, USA).

Measurement of vascular endothelial function: ACUSONG 128XP/10 color Doppler ultrasound diagnostic instrument was used, with 5-10MHZ frequency conversion probe in accordance with the methods of Celemajer[16]. Each patient was synchronously connected with limb electrocardiogram, and the measurement of internal diameter was at the end of diastole. After 10 minutes of rest in supine position, the probe was placed 2 to 5 cm above the elbow joint, showing the long axis section of the radial artery, and the radial diameter D_0 of the radial artery was measured. The cuff of sphygmometer was connected to the lower elbow joint of the forearm at 2 to 3 cm, inflated and pressurized to 300 mmHg for 50 min, and then, quickly deflated. The inner diameter D_1 after reactive hyperemia was measured after 60 to 90s. After a full rest for 10 minutes, 500 μg of nitroglycerin was given sublingually. The radial diameter D_2 was measured again after 4 minutes. All measurements were averaged for 3 cardiac cycles. Endothelial-dependent diastolic (FMD) = $(D_1-D_0)/ D_0 \times 100\%$. Endothelial-independent diastolic (NMD) = $(D_2-D_0)/ D_0 \times 100\%$. Vascular endothelial function was measured by ultrasound specialists.

Statistical Analysis

Statistical Product and Service Solution (SPSS) 18.0 (IBS Inc., Chicago, IL, USA) was used to analyze the data, and GraphPad Prism 6 (La Jolla, CA, USA) was used to draw all the pictures of this experiment. Chi-square test was used to compare the counting data. Mean ± standard deviation was used to express the measurement data. *t*-test was used to analyze the relationship between the two groups. Variance analysis was used for comparison between multiple groups. Pearson correlation analysis was used to analyze the relationship between variables. When $p<0.05$, there was statistical difference.

Results

General Data

There were no significant differences in BMI, age, and WHR of the selected patients (all $p>0.05$) (Table I).

Comparison of TG, ALT and AST Levels Between the two Groups

The levels of TG, ALT and AST in patients with non-alcoholic fatty liver disease were higher than those in the control group, and the difference was statistically significant (all $p<0.05$) (Figure 1).

Comparison of FMD and NMD Levels Between the two Groups

The level of FMD in the test group was significantly lower than that in the control group

Table I. General Information.

Factors	Test group n = 95	Control group n = 90	t/χ^2	*p*
Age	36.7 ± 3.75	35.9 ± 4.23	1.272	0.1746
Course/y	4.49 ± 2.4	5.14 ± 3.04/y	1.604	0.1073
BMI	28.76 ± 3.47	29.14 ± 2.72	1.628	0.4099
WHR	0.81 ± 0.12	0.79 ± 0.14	1.045	0.2974
Gender				0.9439
Male	70 (73.68)	67 (74.44)	0.01390	0.9061
Female	25 (26.32)	23 (25.56)		
Whether smoking			0.02849	0.8660
Yes	55 (57.89)	51 (56.67)		
No	40 (42.11)	39 (43.33)		
Whether eating sweets			0.04470	0.8326
Yes	32 (33.68)	29 (32.22)		
No	63 (66.32)	61 (67.78)		
Family history of liver disease			0.4540	0.5005
Yes	34 (35.79)	28 (31.11)		
No	61 (46.21)	62 (68.89)		

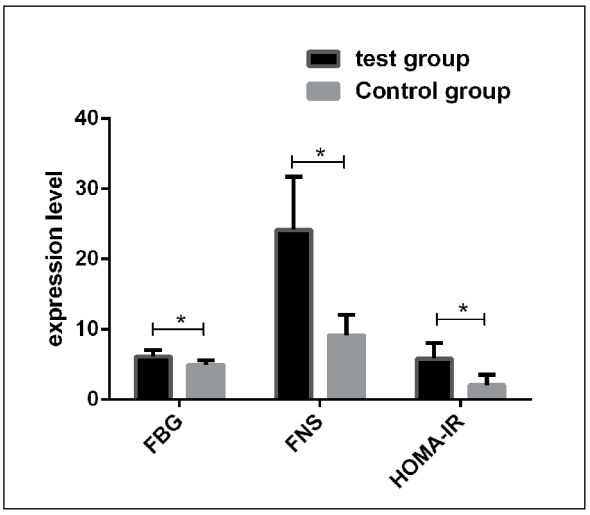

Figure 1. The expression level of TG, ALT and AST in serum of two groups of patients.

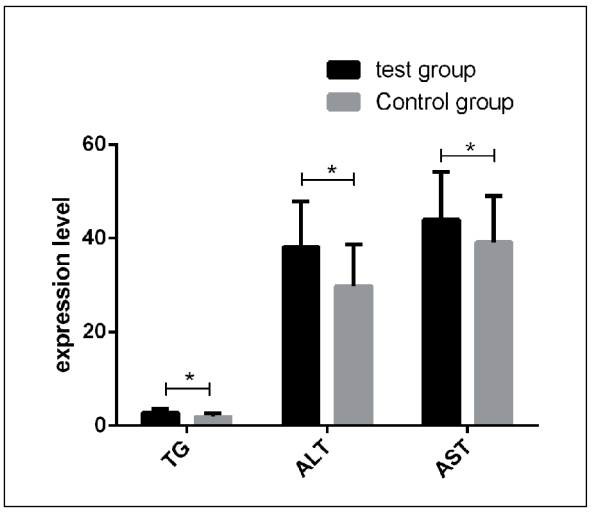

Figure 3. Expression of FBG, FNS and IR in serum of two groups of patients.

(p<0.05). There was no significant difference in the level of NMD between the two groups (p>0.05) (Figure 2).

Comparison of FBG, FNS and IR Between the two Groups

Fasting blood glucose (FBG), fasting insulin (FNS), and insulin resistance (IR) in patients with non-alcoholic fatty liver disease were higher than those in the control group, and the difference was statistically significant (all p<0.05) (Figure 3).

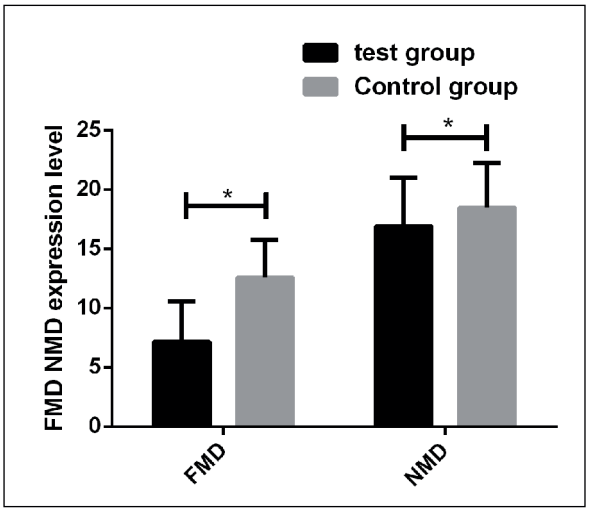

Figure 2. Comparison of FMD levels between the test and control group.

The Expression Levels of ER Markers GRP78, XBP1, CHOP and Caspase-12 in two Groups

RT-PCR results showed that the expression of ER markers GRP78, XBP1, CHOP and caspase-12 in the test group was significantly higher than that in the control group (all p<0.05) (Figure 4).

Correlation Analysis

Pearson correlation analysis showed that the level of ER stress marker protein was correlated with insulin resistance and liver function. GRP78,

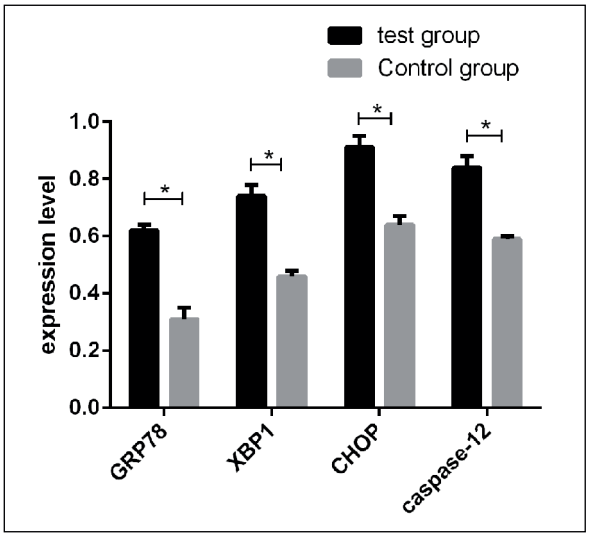

Figure 4. The expression levels of ER marker proteins in two groups.

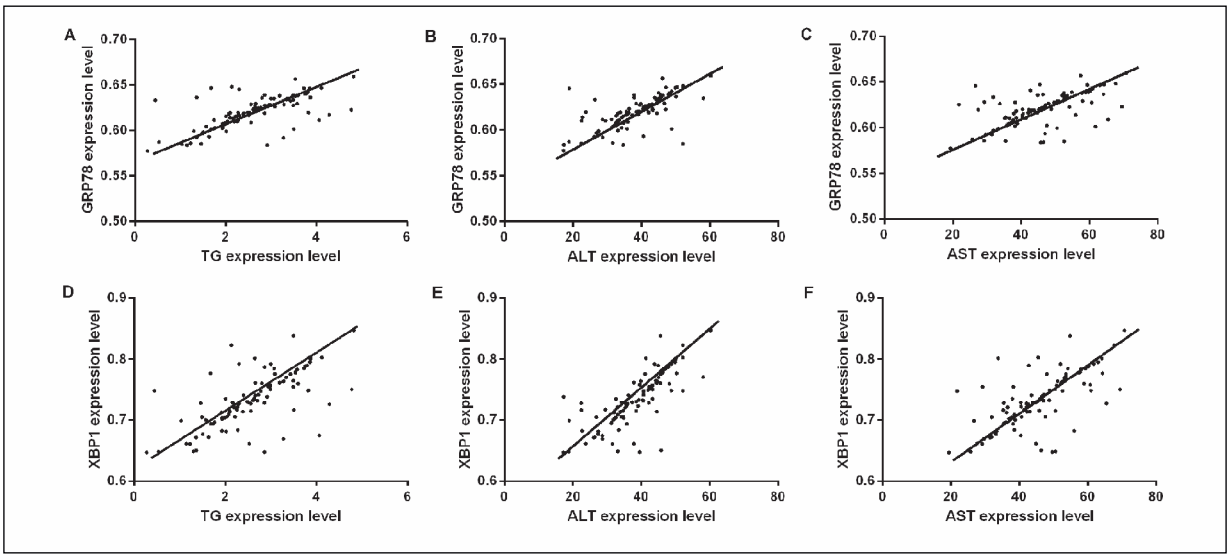

Figure 5. Analysis of correlation between GRP78, XBP1 and TG, ALT and AST in the test group.

XBP1, CHOP and caspase-12 were positively correlated with liver function (all $p<0.05$). GRP78, XBP1, CHOP, and caspase-12 were negatively correlated with flow-mediated dilation (FMD) (all $p<0.05$) (Figures 5-8).

Discussion

Non-alcoholic fatty liver disease can be divided into two categories: primary and secondary. Most non-alcoholic fatty patients are obese and initially asymptomatic, while some patients have symptoms and signs, such as fatigue, indigestion, and pain in the liver area. The accumulation of fat on the surface of the liver can damage hepatocytes. Without timely and effective treatment, there would be inflammation and fibrosis[17]. For the first time, we analyzed the relationship between non-alcoholic fatty liver disease and liver function, insulin resistance, and vascular endothelial function. The idea of combined detection may be used in the diagnosis and treatment of non-alcoholic fatty liver disease.

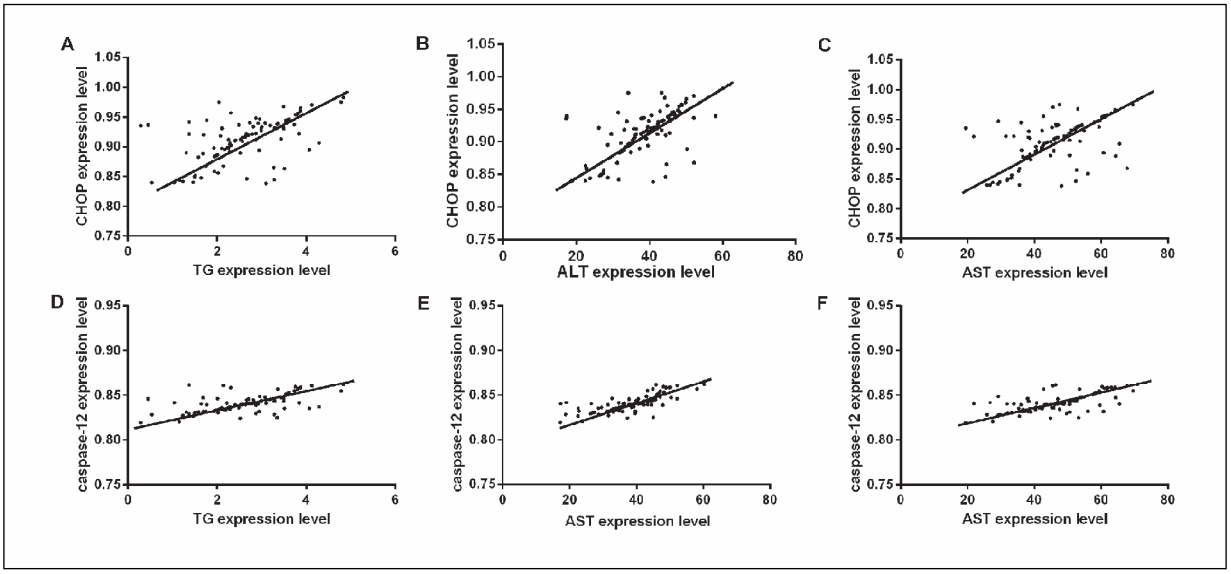

Figure 6. Analysis of correlation between CHOP, caspase-12 and TG, ALT and AST in the test group.

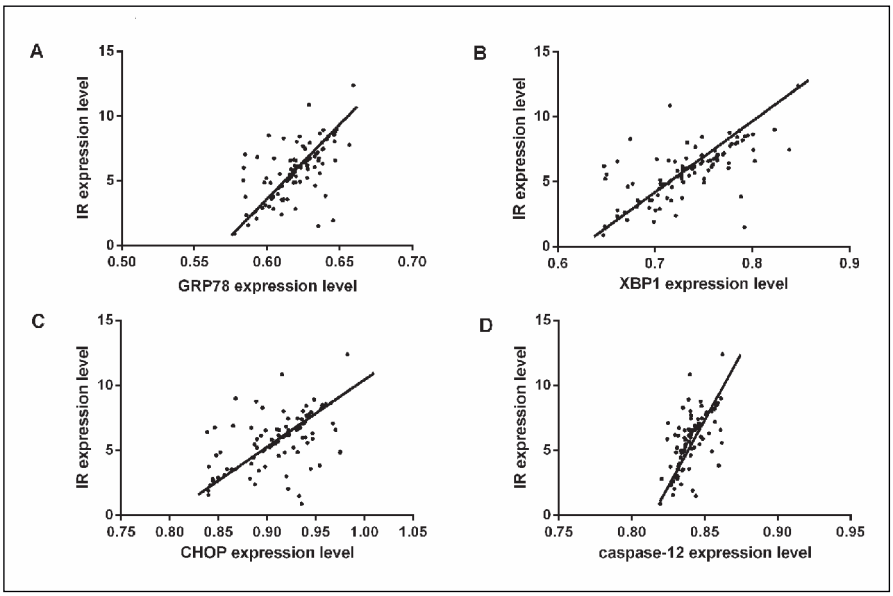

Figure 7. Analysis of correlation between ER stress marker protein and insulin in the test group.

ERS response is an important mechanism of cell resistance to stress. Moderate response is helpful to protect cells and maintain survival, but severe ER stress for a long time may damage the function of ER, causing cell dysfunction, and then leading to cell apoptosis. ERS involves unfolded protein reaction (UPR) and imbalance of Ca^{2+} in ER, which activates caspase-12 and induces significant increase of molecular chaperones such as CHOP78, GRP94, GADD34, GADD45A, CHOP and CRT. It leads to a series of combined reactions to induce cell damage and apoptosis[18]. Unfolded protein response (UPR) is mediated by an endoplasmic reticulum chaperone GRP78 and three stress-responsive receptor proteins, distributed by PERK, ARF6 and IREI[19]. GRP78, also known as BiP (immunoglobulin heavy chain binding protein), is the main molecular chaperone in ER. It is also a key molecule in ER stress response because of its low expression level in normal tissues. If there were ERS, the unfold protein accumulates in the endoplasmic reticulum, causing the dislocation of GRP78 from the three transmembrane proteins, and then, binding GRP78 to the folded protein. The dissociated receptor proteins IRE1 and PERK are

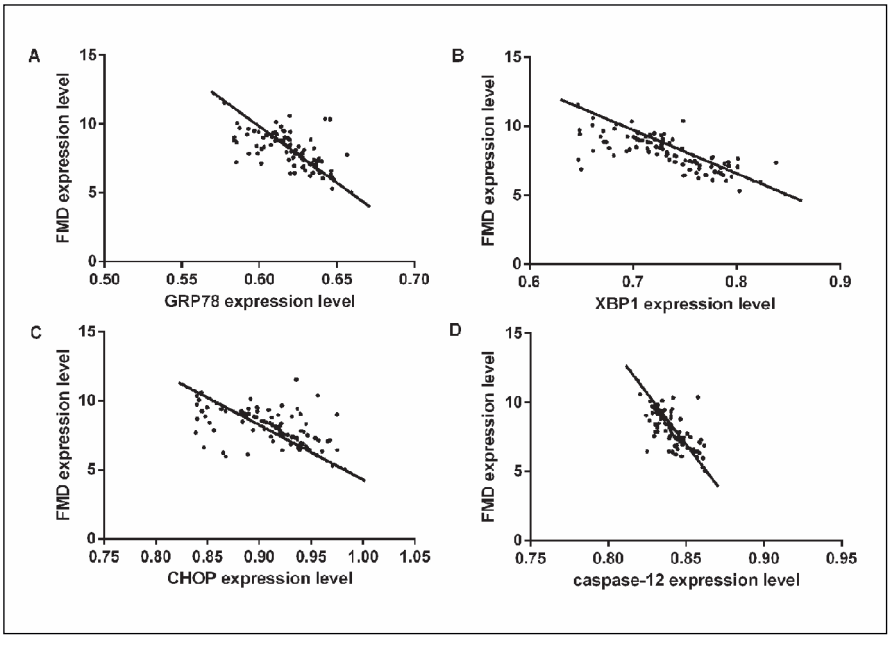

Figure 8. Analysis of correlation between ER stress marker protein and vasodilation function in the test group.

transformed into ER stress response signals by transmembrane phosphorylation[20]. The change in expression of XBP-1 directly affects the intensity of ERS response and affects the transmission of insulin signaling[21,22]. CHOP belongs to the C/EBP transcription factor. When ERS reacts, the expression of CHOP increases significantly and induces apoptosis. Caspase-12, located on the cytoplasmic surface of the ER, is a specific mediator of ERS-induced apoptosis[23-25]. Its activation enables ERS to independently induce apoptosis without relying on other pathways. Therefore, GRP78, XBP-1, CHOP, and caspase-12 are often used as standard proteins for ER stress.

In this study, patients with NAFLD and obese non-fatty liver were first selected. The expression of XBP-1, CHOP, caspase-12 and other central markers in NAFLD patients were significantly higher than those in the control group by RT-PCR, while GRP78 showed an upward trend. These results suggested that endoplasmic reticulum stress exists in non-alcoholic fatty liver disease. Scholars[26] have shown that ERS can induce IR in peripheral tissues, including liver, muscle, and fat. IR refers to the decrease of the efficiency of insulin in promoting glucose uptake and utilization for various reasons. The compensatory excessive secretion of insulin in the body produces hyperinsulinemic hypoglycemia to maintain the stability of blood sugar. It is an abnormal pathophysiological state, and it is a sign of many clinical diseases or symptoms, especially the common endocrine metabolic disease[27]. The results showed that the IR of the test group was significantly higher than that of the control group. IR can result in the hydrolysis of fat stored in cells, the elevation of free fatty acids (FFA) in blood and the entry into extra-adipose tissues, including liver tissues. The lipids after meals are also absorbed by the liver in the form of fatty acids, which increase the synthesis of liver TG and store in hepatocytes, leading to liver lipid deposition. Increased FFA can inhibit insulin signal transduction and aggravate IR[28]. In addition, IR can also cause abnormal secretion of adipose-derived cytokines. It leads to visceral obesity and inflammatory state, inhibit FFA metabolism, damage the antioxidant and anti-apoptotic of liver cells, and promote the development of fatty liver disease[29].

The results showed that endothelium-dependent vasodilation in NAFLD patients was significantly lower than that in the control group under ER stress. The reactive hyperemia experiment[30] is a common clinical method for judging vascular endothelial dysfunction. The shear force produced by the sudden increase of blood flow acts on the vascular endothelium, stimulates the secretion of carbon monoxide, and dilates the blood vessels. When the vascular endothelial dysfunction occurs, the blood flow-mediated relaxation function decreases, which is endothelium-dependent. Nitroglycerin, as an exogenous carbon monoxide donor, can directly act on the subvascular smooth muscle to dilate blood vessels. It indicates that FMD is decreased in NAFLD patients, but the function of subendothelial smooth muscle is not affected. Endothelial cells are one of the target organs of insulin action. Insulin can stimulate vascular endothelial cells to release more carbon monoxide and cause vasodilation. However, in insulin resistance, tissue sensitivity to insulin is decreased, insulin-mediated endothelial cell-dependent vasodilation is impaired, and endothelial-derived nitric oxide production is decreased[31]. In addition, lipid peroxidation, oxidative stress and low-grade inflammation caused by insulin resistance are all the causes of aggravating endothelial injury.

Insulin signaling pathway is a cascade of tyrosine phosphorylation: the self-activation of insulin receptor tyrosine kinase followed by tyrosine phospholipid acidification of insulin receptor substrate-1 (IRE-1). Kaneto et al[32] found that ERS could activate inositol requiring-1α (IRE-1α), leading to c-JUN NH2-terminal kinase (JNK) signaling pathway activation. Ozcan et al[33] induced liver cell ERS by tunicamycin and found that phosphorylation of Akt stimulated by insulin and phosphorylation of IRS-1 serine were enhanced. Compared with tyrosine phosphorylation, serine phosphorylation of insulin receptor or its downstream signal ligand prevents insulin signal transduction, reducing insulin sensitivity in peripheral tissues, leading to IR[34]. In this study, the expression of ER stress marker protein XBP-1 in the test group was higher than that in the control group, indicating that the IRE-1 and α-JNK signaling pathways of hepatocytes in the test group changed, affecting insulin signal transduction and insulin resistance.

Conclusions

Through the results of this study, it is speculated that insulin resistance is closely related to the occurrence of ER stress in non-alcoholic fatty

liver disease. ER stress may reduce the sensitivity of peripheral tissues to insulin by insulin signal transduction, resulting in insulin resistance and the formation of non-alcoholic fatty liver disease. However, further studies are still needed to investigate the mechanism of vascular endothelial dysfunction in patients with NAFLD. Insulin resistance is one of the factors that induce and aggravate the inhibition of endothelial function. Improving insulin resistance in patients with NAFLD may reduce the risk of cardiovascular disease.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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