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Original Article

# Effect of aerobic exercise on GRP78 and ATF6 expressions in mice with non-alcoholic fatty liver disease

Junhan Li [a,*], Lei Huang [b], Wei Xiong [c], Chun Gu [c], Shuling Zhang [a], Xiali Xue [a]

[a] School of Sports Medicine and Health, Chengdu Sport University, Chengdu, Sichuan Province, China
[b] Department of Exercise Rehabilitation, Sichuan Provincial Orthopedic Hospital, Chengdu, Sichuan Province, China
[c] Department of Hepatobiliary Surgery, Sichuan Provincial People's Hospital, Chengdu, Sichuan Province, China

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ABSTRACT

Nonalcoholic fatty liver disease (NAFLD) is a prevalent medical condition with an ever-growing trend. Although multiple intracellular mechanisms are involved, endoplasmic reticulum (ER) stress has been demonstrated to play a significant role in the genesis and progression. Most of the research supports the advantages of exercise for NAFLD. However, little is known about the molecular mechanism(s) that underpin the effectiveness of exercise training in NAFLD. This study aimed to identify how aerobic exercise affected hepatic ER stress in a mouse NAFLD model. In this study, the mice were fed either a standard diet (SD) or a high-fat diet (HFD) for 17 weeks. HFD mice were trained on a treadmill during the last eight weeks. All animals were tested for serum levels of biochemical assays, protein expression, and gene expression. The hematoxylin and eosin, Oil red O, and immunohistochemistry staining were also performed. The results indicated that a high-fat diet generated NAFLD, with serum lipid disruption and hepatic function impairment, and increased GRP78 and ATF6 expressions. However, aerobic training reversed the majority of these alterations. It is concluded that NAFLD appears to be associated with hepatic ER stress response, and aerobic exercise mitigates NAFLD via lowering ER stress proteins GRP78 and ATF6.

## Introduction

Nonalcoholic fatty liver disease (NAFLD) is characterized by steatosis in more than 5% of hepatocytes despite little or no alcohol consumption.[1] NAFLD is the most prevalent liver disease worldwide, and NAFLD prevalence has been increasing in parallel with the prevalence of obesity and diabetes.[2] Due to the challenges of coronavirus disease 2019 pandemic, NAFLD continued to evolve in recent years rapidly.[3] As a result, it is critical to adopt appropriate measures to mitigate NAFLD.

There is no approved clinical pharmacological treatment to treat NAFLD.[4] The current treatment options are lifestyle modifications involving food and exercise.[5] Physical inactivity is a contributor to NAFLD development and progression.[6] Gerber et al. demonstrated that NAFLD patients had the lowest quartile of moderate physical activity and moderate to intense physical activity.[7] Zelber-Sagi et al. discovered that NAFLD patients often engage in fewer aerobic, resistance, or other physical activities.[8] As a result, increased physical activity has become one of the most commonly suggested treatments for NAFLD.[9] Exercise training reduces the frequency and severity of NAFLD in animals[10] and humans.[11] However, little is known about the molecular mechanism(s) behind the benefits of exercise training in NAFLD.

The endoplasmic reticulum (ER) is the key organelle for protein synthesis and folding, maintaining a balance between the two.[12] However, under some pathological conditions in which the rate of protein synthesis exceeds the folding capability, unfolded proteins begin to accumulate in ER, causing a disruption in ER homeostasis, inducing ER stress (ERS), activating an unfolded protein response (UPR).[13] The UPR is carried out by the main chaperones glucose-regulating protein 78 kDa (GRP78) and three ER transmembrane receptors, which include activating an inositol-requiring enzyme 1 α (IRE1α), a protein kinase-R-like ER kinase (PERK), and a transcription factor 6 (ATF6).[14] Each of these proteins activates specific pathways that enhance the accurate folding capacity of proteins and accelerate the degradation of misfolded proteins.[15]

GRP78 is a marker of ER stress, and the elevated GRP78 level suggests a severe protein misfolding condition. GRP78 mRNA was decreased in adipose tissue of gastric bypass patients, implying that the relationship between obesity-related ER stress and metabolism dysfunction exists in humans.[16] A recent study showed that GRP78 (+/-)mice fed a high-fat

* Corresponding author. School of Sports Medicine and Health Chengdu Sport University, No 2, Tiyuan Road, Wuhou District Chengdu, Sichuan, 610041, China.
*E-mail address:* junhanli@cdsu.edu.cn (J. Li).

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**Abbreviations**

| | |
|---|---|
| **NAFLD** | Nonalcoholic fatty liver disease |
| **ERS** | Endoplasmic reticulum stress |
| **SD** | Standard diet |
| **HFD** | High-fat diet |
| **SD + Ex** | Standard diet with exercise |
| **HFD + Ex** | High-fat diet with exercise |
| **TG** | Triglyceride |
| **TC** | Total cholesterol |
| **HDL-c** | HDL-cholesterol |
| **LDL-c** | LDL-cholesterol |
| **AST** | Aspartate amino-transferase |
| **ALT** | Alanine amino-transferase |
| **LIT** | Low-intensity training |
| **HIT** | High-intensity training |
| **UPR** | Unfolded protein response |
| **GRP78** | Glucose-regulated protein 78 |
| **PERK** | Protein kinase R-like ER kinase |
| **eIF2α** | Eukaryotic translation initiation factor 2α |
| **IRE1** | Inositol-requiring enzyme 1 |
| **ATF6** | Transcription factor 6 |
| ***SEM*** | Standard error of the mean |
| **ER** | Endoplasmic reticulum |
| **AMPK** | Adiponectin and adenosine monophosphate-activated protein kinase |
| **SREBP-1c** | Sterol regulatory element-binding protein-1c |
| **PPARα** | Peroxisome proliferator-activated receptor α |

diet for 30 weeks demonstrated less weight gain, greater insulin sensitivity, and improved glucose homeostasis independent of adiposity.[17] ATF6 is regarded as an ER stress-activated transcription factor. One research found that ATF6 physically interacts with peroxisome proliferator-activated receptor α (PPARα), enhances the transcriptional activity of PPARα, and triggers activation of PPARα downstream targets in diet-induced insulin-resistant mice.[18] Another research found that liver-specific knockout of ATF6 exacerbated HFD-induced hepatic steatosis by MTOR-mediated down-regulation of autophagy.[19] However, few studies have reported the effect of exercise on hepatic GRP78 And ATF6 expressions in NAFLD.

Studies on the influence of persistent physical exercise on ER stress have been undertaken in various tissues. For example, increased UPR is observed in hypothalamus (ATF6 and GRP78), hippocampus (ATF6 and GRP78), and cortex (ATF6) in the mouse brain after three weeks of voluntary running wheel exercise.[20] This study aimed to investigate the influence of exercise on GRP78 and ATF6 expressions in the liver. It is anticipated that aerobic exercise may help reduce NAFLD via reducing ER stress proteins GRP78 and ATF6.

## Materials and methods

### *Experimental animals and treatments*

The experiments were conducted on 40 male C57BL/6 mice (age = 8 weeks). The mice were housed in collective cages (four mice per cage), with a controlled room temperature (25 ± 1) °C, relative humidity (50% - 60%), and a light/dark cycle of 12/12 h. The mice had unrestricted access to food and water. The Institutional Animal Care and Use Committee of Chengdu Sport University approved the study protocols, which were carried out in compliance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication, 8th edition, 2011).

All animals were fed the standard diet during the one-week acclimatization period. At week 2, all animals were randomly divided into four groups, namely, the standard diet (SD) group, standard diet with exercise (SD + Ex) group, high-fat diet (HFD) group, and high-fat diet with exercise (HFD + Ex) group, with ten mice in each group. During the entire experiment period, groups SD and SD + Ex were given the standard diet (SD, 10% total calories from fat, 20% total calories from protein, and 70% total calories from carbohydrate), while groups HFD and HFD + Ex were fed a high-fat diet (HFD, 60% fat calories, 20% carbohydrate calories, and 20% protein calories in total calories).[21] The compositions of the high-fat and standard diets are given in Table 1. Jiangsu Synergy Pharmaceutical Biotechnology Co., Ltd. (Nanjing, China) provided the SD and HFD. At week 10, groups SD + Ex and HFD + Ex began an eight-week treadmill exercise regimen after a one-week acclimatization period.

**Table 1**
The composition of the high-fat and standard diet.

| Standard diet (%) | High-fat diet (%) |
|---|---|
| Protein 21% | Standard diet 67.7% |
| Fat 3.69% | Ghee 8.3% |
| Carbohydrate 32.5% | Hydrogenated oil 4.05% |
| Crud fibre 5.5% | Soybean oil 0.85% |
| | Sodium cholate 0.8% |
| | Cholesterol 1.0% |
| | Sugar 17.3% |

### *Exercise training protocol*

The aerobic training program was developed using the treadmill exercise model of NAFLD mice[22] and integrating it with the actual exercise capacity of mice. The animals in groups SD + Ex and HFD + Ex were subjected to treadmill adaptation training for one week, beginning with an initial speed of 8 m/min × 10 min at 0° slope and gradually increasing the speed and time to 12 m/min × 60 min in five days. Formal training was done for eight weeks at a pace of 12 m/min, a slope of 0°, 60 min per day, and five days per week. The speed was about 70% of the maximum speed of the mice. The peak velocity was determined as the velocity of the last stage completed by the animals. An incremental exercise test on a flat treadmill mill estimated maximum velocity (no slope). The initial velocity was 8 m/min, raised by 2 m/min every 2 min until the animals could not run.[23] Animals who had not been physically active were placed on treadmills at the same frequency and duration as the physically active mice. All animals fasted for 12 h and the final exercise session was performed 48 h before euthanasia.

### *Sample preparation*

All animals were sacrificed at the end of the experiment following anesthesia with a single intraperitoneal injection of pentobarbital sodium. Before analysis, blood samples were withdrawn, and the serum was separated and refrigerated at −20 °C. The liver index was calculated by collecting and weighing liver tissue samples (liver index [%] = liver weight/body weight × 100%). The liver tissues were divided into two halves. One section was fixed in 10% formalin and typically embedded in paraffin for hematoxylin and eosin (HE) staining. Another portion was snap-frozen in liquid nitrogen for Oil Red O staining and stored at −80 °C for later examination.

### *Histopathological examination*

The mice's liver tissues were fixed in 10% paraformaldehyde, embedded in paraffin, sectioned on 4–5 μm thick slices, and stained with

**Table 2**
Primer synthesis sequence.

| Genes | Forward primer (5′-3′) | Reverse primer (5′-3′) |
|---|---|---|
| GRP78 | GCCGAGACAACACTGACCTGGACACT | CCACCGTGCCCACATCCTCCTTCTT |
| ATF6 | CGGCTTCCTCCAGTTGCTCCATCTC | TCCAGGACCAGTGACAGGCTTCTCTT |
| β-actin | GAAGATCAAGATCATTGCTCC | TACTCCTGCTTGCTGATCCA |

hematoxylin and eosin (H & E). The slides were analyzed and respective sections were captured at 400 magnifications using a light microscope (Olympus BX-50 Microscope, Leica Microsystems, Germany). NAFLD score method developed by the Pathology Committee of Non-Alcoholic Steatohepatitis Clinical Research Network[24] for the microscopic evaluation was used. The oil red O staining approach was used to observe the lipid droplets in the hepatocytes. The tissue slices were incubated with Oil Red reagents, and respective sections were photographed with a light microscope (Olympus BX-50 Microscope, Leica Microsystems, Germany). The density of lipid droplets was calculated by measuring the area of lipid droplets with Image-Pro Plus software (Version 6.0, Media Cybernetics, Inc., MD, USA).

*Biochemical measurements*

The levels of triglycerides (TG), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-c), high-density lipoprotein cholesterol (HDL-c), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) were measured using an Olympus AU2700 Autoanalyzer (Olympus, Japan).

*Western blot*

RIPA + PMSF (100:1) buffer was used to extract liver proteins, followed by measuring using the Bradford method (Santa Cruz, USA). Under decreasing circumstances, equal amounts of 50 μg of soluble protein were loaded on 12.5% SDS-PAGE and then transferred to PVDF membranes. Non-specific proteins were blocked for 1 h at room temperature with 5% non-fat dry milk. The membranes were then incubated with anti-GRP78 (1:1 000 dilution, sc-376768, Santa Cruz, USA) and ATF6 (1:1 000 dilution, ab37149, Abcam, United Kingdom) overnight at 4 °C, with β-actin (1:1 000, Cell Signaling Technology, USA) serving as a control. After the incubation with HRP-conjugated secondary antibody (1:4 000, Santa Cruz, USA) for 1 h at room temperature, the immunoblot was then observed with the ECL Plus chemiluminescence reagent kit (Amersham Bioscience, PA, USA) and analyzed with Gel-pro 32 software (Media Cybernetics, MD, USA).

*Quantitative real-time polymerase chain reaction (qRT-PCR)*

Total RNA was isolated from liver tissue using Trizol reagent (Invitrogen, Carlsbad, CA, USA), and cDNA was synthesized with the HiScript

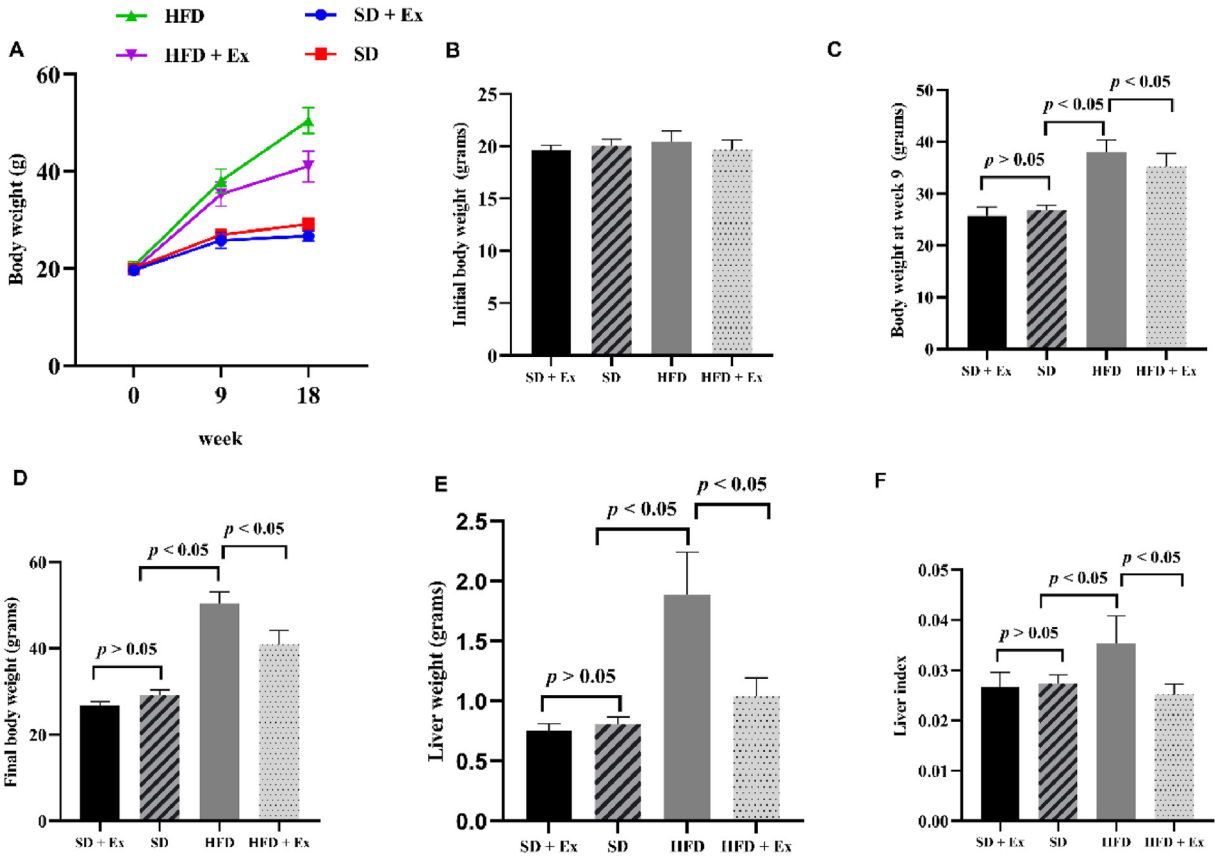


**Fig. 1.** Effect of aerobic exercise on body weight, liver weight and liver index. body weight curve (A); initial body weight (B); body weight in the ninth week (C); final body weight (D); liver weight (E); liver index (F). All values are mean ± *SEM* ($n$ = 9–10 for each group). SD: standard diet, HFD: high-fat diet, SD + Ex: standard diet with exercise, HFD + Ex: high fat diet with exercise.

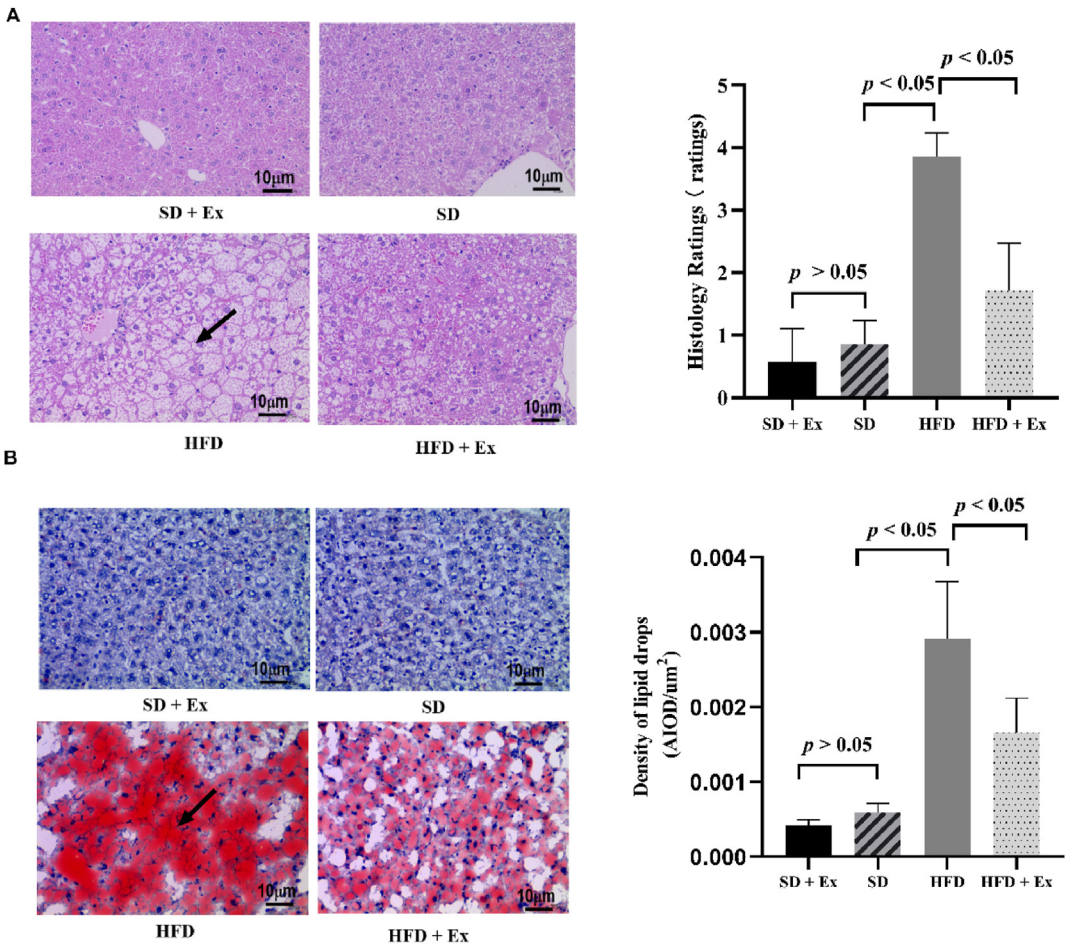


**Fig. 2.** Effect of aerobic exercise on liver morphology. H & E staining and Quantification of histology ratings (A); Oil Red O staining and Quantification of Oil Red O by Image-Pro Plus software (B). Scale bars representing 10 μm, × 400. All values are mean ± *SEM* ($n = 6$ for each group). SD + Ex: standard diet with exercise, SD: standard diet, HFD: high-fat diet, HFD + Ex: high fat diet with exercise.

II Q RT SuperMix (Invitrogen, Carlsbad, CA, USA) (Vazyme, Nanjing, China). The AceQ qPCR SYBR Green Master Mix was used to perform quantitative real-time PCR (Vazyme, Nanjing, China). Each experiment was repeated three times. The relative expression of the target genes was determined using the $2^{-\Delta\Delta Ct}$ method. GenScript Co., Ltd. was responsible for designing and producing the primers (Nanjing, China). Table 2 lists the primers that were utilized.

### *Immunohistochemistry*

Liver tissue sections (5 μm) were mounted on the coated slides. Tissue sections were deparaffinized and rehydrated, then subjected to antigen retrieval for 1 h at room temperature incubation with 3% bovine serum albumin to inhibit non-specific binding. Anti-GRP78 (1/50 dilution, sc-376768, Santa Cruz, USA) and anti-ATF6 (1/100 dilution, ab37149, Abcam, United Kingdom) primary antibodies were used to detect GRP78 and ATF6 expression. The sections were then developed for 1–3 min in a diaminobenzidine solution (DAB). The slides were inspected using a photomicroscope (Olympus Shinjuku, Tokyo, Japan), and the images were quantified using Image J software (ImageJ 1.5, NIH, USA). The findings came from comparing the average integrate optical density.

### *Statistical analysis*

The data are displayed as mean ± *SEM* (standard error of the mean,*SEM*). SPSS software was used to analyze all data (version 25.0, Chicago, IL). The data were statistically evaluated using two-way ANOVA to detect significant main effects of exercise (exercise vs. non-exercise) and diet (SD vs. HFD). Tukey's multiple-comparison post hoc analysis evaluated differences when significant main effects were identified. The $p < 0.05$ was considered statistically significant.

## Results

### *Body weight, liver weight, and liver index*

The body weight and liver index results are presented in Fig. 1. In all groups, there was a progressive increase in body weight growth. Aerobic exercise effectively lowered body weight, liver weight, and liver index (liver weight-to-body weight ratio). At the end of the experiment, the body weight was greater in the HFD group than in the SD group ($p < 0.05$) and lower in the HFD + Ex group than in the HFD group ($p < 0.05$). The liver index was higher in the HFD group compared to the SD group ($p < 0.05$) and lower in the HFD + Ex group compared to the HFD group ($p < 0.05$). However, there was no significant difference in the SD + Ex group compared to the SD group ($p > 0.05$).

### *Histopathological examination*

Hematoxylin and eosin (H & E) (Fig. 2A) shows that the SD group exhibited a normal, transparent, and regular liver lobular architecture, with numerous basophilic granules in the hepatocytes and just a single layer of cells surrounding the central vein. The liver tissues in the HFD group had severe, extensive lipid vacuoles inside the parenchyma cells,

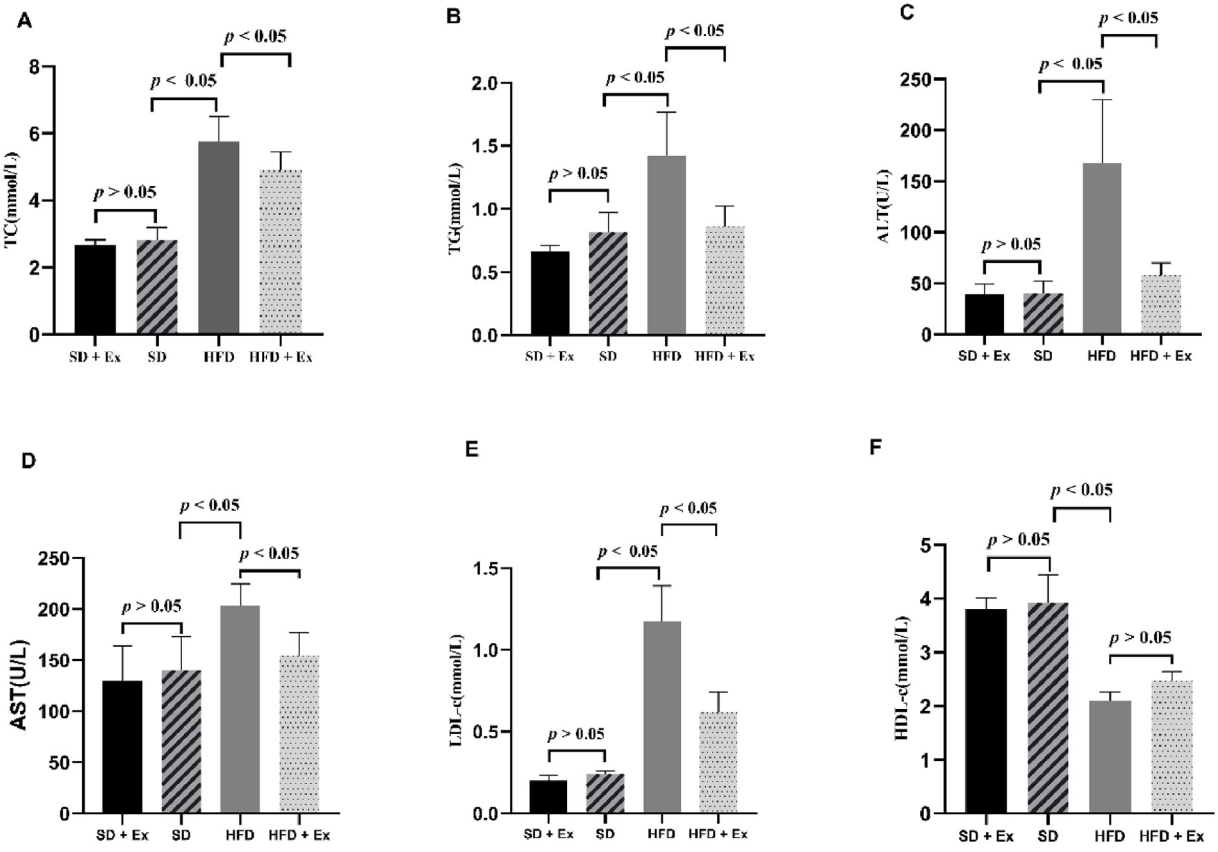


**Fig. 3.** Effect of aerobic exercise on serum lipids profiles and liver function enzyme in mice. The serum lipids profiles and liver function enzymes are presented. TC (A); TG(B); ALT (C); AST (D); LDL-c(E); HDL-c(F). All values are mean ± *SEM* ($n$ = 6–7 for each group). TC: total cholesterol, TG: triglycerides, ALT: alanine aminotransferase, AST: aspartate aminotransferase, LDL-c: low-density lipoprotein cholesterol, HDL-c: high-density lipoprotein cholesterol. SD + Ex: standard diet with exercise, SD: standard diet, HFD: high-fat diet, HFD + Ex: high fat diet with exercise.

infiltrating lymphocytes, and centrilobular liver necrosis. Compared to the HFD group, the HFD + Ex group avoided histopathological abnormalities and alleviated micro-vesicular fatty changes ($p < 0.05$).

Oil Red O staining (Fig. 2B) displays that the HFD group accumulated lipid droplets and a higher density of lipid droplets than the SD group. Aerobic exercise training reduced lipid droplet density in the HFD + Ex group compared to the HFD group ($p < 0.05$). However, no significant difference existed in histopathological alterations between SD + Ex and SD groups ($p > 0.05$).

*Serum lipid profile and liver enzyme analysis*

The serum concentrations of TG, TC, LDL-c, AST, and ALT were higher in the HFD group than in the SD group (Fig. 3A–E), which were reduced by aerobic exercise (Fig. 3A–E). The serum concentration levels of HDL-c were lower in the HFD group than in the SD group (Fig. 3F), which remained unchanged by aerobic exercise (Fig. 3F). However, there were no significant differences existed in all above indicators between groups SD + Ex and SD (Fig. 3A–F).

*GRP78 and ATF6 expressions*

The HFD group expressed higher levels of the proteins GRP78 and ATF6, as well as higher levels of mRNAs encoding GRP78 and ATF6 than the SD group (Fig. 4A–E). Exercise training reversed these diet-induced changes (Fig. 4A–E). Meanwhile, the immunohistochemical analyses showed that the optical density of GRP78 and ATF6 were higher in the HFD group than that in the SD group (Fig. 4F and G), and lower in the HFD + Ex group than that in the HFD group (Fig. 4H and I). However, all the above parameters had no significant differences between groups SD + Ex and SD (Fig. 4A–I).

## Discussion

Developing an effective treatment strategy for NAFLD has received exceedingly challenging as the clinical burden of NAFLD has become more apparent. Physical activity has been found to improve the condition of NAFLD.[25] This investigation discovered that HFD increased the expressions of ER stress-related proteins such as GRP78 and ATF6. GRP78 and ATF6 expression were reduced by aerobic exercise, resulting in a reduction in ER stress and NAFLD improvement.

Numerous pieces of research imply that exercise improves NAFLD because it results in weight loss or average weight.[26] Severely obese women who increase their physical activity level can improve their lipoprotein profile regardless of whether or not they reduce fat mass.[27] As expected, the findings of this investigation revealed that high-fat diet (HFD) feeding had a substantial impact on raising the body weight of experimental animals. Simultaneously, aerobic exercise training lowers the weight increase of the high-fat-fed mice.

Several pathways for exercise-induced protective benefits have been postulated, including lower TC, LDL-c, and higher HDL-c levels.[28] One research found that four weeks of regular daily aerobic exercise could lower TC, TG, and LDL-c levels, but the short-term intervention was insufficient to increase HDL-c levels.[29] Our findings revealed that HFD feeding was more effective than SD at increasing plasma TG, TC, and LDL-c levels. Aerobic exercise improved lipid profiles by lowering TG, TC, and LDL-c in the blood. Furthermore, as validated by our investigation, moderate-intensity aerobic exercise for 24 weeks enhanced liver function, as demonstrated by the presence of ALT and AST in NAFLD patients.[30]

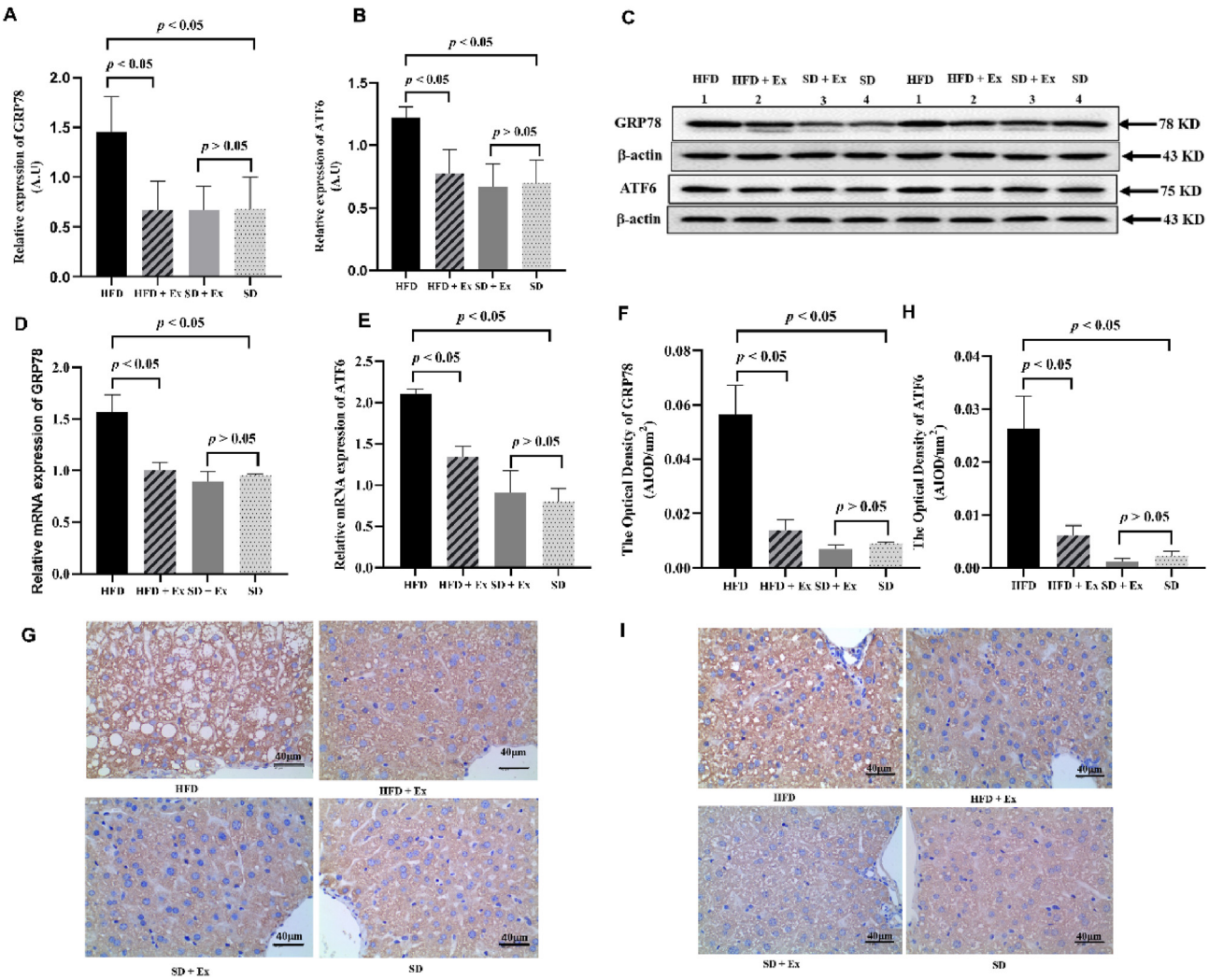


**Fig. 4.** Effect of the aerobic exercise on GRP78 and ATF6 expressions in mice. Western blot analysis and densitometry (A–C). relative expression of GRP78 (A); Relative expression of ATF6 (B); immunoblots against the indicated proteins in liver tissue (C). Quantitative real-time polymerase chain reaction (qRT-PCR) analysis was used to detect the relative mRNA expression of GRP78 (D) and ATF6 (E). Immunohistochemistry analysis was used to obtain representative immunostaining images and the optical density of GRP 78 (F–G) and ATF6 (H–I). All images were obtained from the ventricular septum (bar length = 40 μm) and were magnified at 400 ×. All values are mean ± *SEM* ($n$ = 6 for each group). HFD: high-fat diet, HFD + Ex: high fat diet with exercise, SD + Ex: standard diet with exercise, SD: standard diet.

The possible explanations for the effect of endurance exercise on NAFLD are as follows. The pathways of fatty acid oxidation and fatty acid synthesis have pivotal roles in hepatic steatosis. Adiponectin and adenosine monophosphate-activated protein kinase (AMPK) are associated with fatty acid oxidation in the liver.[31] Exercise decreases triglyceride synthesis by increasing adiponectin[32] and AMPK activation.[33] Exercise also reduces sterol regulatory element-binding protein-1c (SREBP-1c) levels in the liver.[34] Thus, exercise has a protective effect on fatty liver via activating the AMPK pathway and/or repressing the SREBP-1c pathway. Moreover, it has been reported that exercise-induced antioxidant enzymes.[35] In addition, exercise may improve NAFLD by enhancing autophagy.[36] Exercise reduces oxidative stress and hepatic gluconeogenesis,[37] which is also a possible mechanism for the effect of endurance exercise on NAFLD.

Evidence suggests that ER stress plays an important role in the development of NAFLD.[38] The induction of ER stress was first described in the livers of genetic and diet-induced models of non-alcoholic steatohepatitis.[39] Since then, these findings have been confirmed in mice fed a methionine-choline-deficient diet exhibiting hepatic steatosis without obesity[40] and in other obese animal models.[41,42] Moreover, ER stress acts indirectly on liver triglyceride accumulation by promoting insulin resistance in both the liver and adipose tissue.[43] In addition, ER stress may lead to numerous intracellular pathways including hepatic steatosis, systemic inflammation, and hepatocyte cell death, all of which are important in the pathogenesis of NAFLD.[44] Despite rapid growth in the field of ER stress research in the context of NAFLD, the exact contribution of the ER stress response to the pathogenesis of NAFLD remains to be fully elucidated.

NAFLD pathophysiology is thought to be caused by ER stress.[45] A recent study found that ATF6 and GRP78 expressions were elevated in NAFLD generated by a high-fat diet.[46] Six-week knee loading, a novel form of physical stimulation, was found to reduce hepatic GRP78 expression in a mouse model of NAFLD.[47] The hepatic ATF6 in mice was reduced by eight weeks of running overtraining regimens, including downhill, uphill, and without an incline.[48] However, GRP78 expression remained unchanged in the liver of HFD mice after six weeks of endurance treadmill exercise.[23] Lifelong exercise training prevented the age-related increase in hepatic GRP78 protein but not in ATF6 protein.[49] This study found that (a) ER stress indicators increased in NAFLD animals, and (b) eight weeks of aerobic exercise lowered GRP78 and ATF6 protein levels in HFD mice but not in SD mice. These seemingly contradicting findings (upregulations and downregulations of GRP78 and ATF6 or no change) imply that the impact of physical exercise on GRP78 and

ATF6 may be associated with different types, intensities, duration of exercise, and even a diverse diet.

Finally, aerobic exercise reduced ER stress and alleviated NAFLD in mice. Since ER stress is at the root of many disorders, future research should concentrate on understanding the pathways involved in hepatic ER stress. The association between NAFLD and ER stress must be examined in greater depth for future studies.

## Submission statement

All authors have read and agree with manuscript content. While this manuscript is being reviewed for this journal, the manuscript will not be submitted elsewhere for review and publication.

## Ethical approval statement

The animals were housed at a controlled room temperature (25 ± 1) °C, relative humidity (50%-60%), and a light/dark cycle of 12/12 h. Food and water were provided ad libitum during experimental period. All procedures were performed in accordance with the guidelines established by the Animal Care and Committee Guidelines of Chengdu Sport University.

## Authors' contributions

JHL had conceived, carried out the experiments and wrote the manuscript. LH had revised and edited the manuscript. WX and CG had reviewed the manuscript. SLZ and XLX had analyzed the data. All authors have read and agreed to the published version of the manuscript.

## Conflict of interest

The authors of this paper declare that they have no direct or indirect interests that are in direct conflict with the conduction of the study.

## Acknowledge statement

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