





OPEN Exercise ameliorates nonalcoholic fatty liver disease by reducing the IGFBP5 to IGF1 ratio to activate AMPK pathway

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Insulin-like growth factor-binding protein 5 (IGFBP5) is associated with nonalcoholic fatty liver disease (NAFLD). IGFBP5 has a higher binding affinity to IGF1 and modulates its effects in serum. However, the effect of exercise on its expression remains unclear. Hence, this study investigated whether exercise regulated hepatic and circulating IGFBP5 levels to ameliorate NAFLD. The NAFLD mice were regularly trained for 12 weeks on a treadmill at a 0% grade. Serum levels of IGFBP5, total IGF1, and free IGF1 were detected using proteome analyses and ELISA. Critical cytokines, such as IRS1, Akt, and AMPK α , and their phosphorylation levels mediated by IGFBP5/IGF1 were confirmed via Western blotting. The results indicated that exercise remarkably alleviated HFD-induced NAFLD and insulin resistance. Although exercise lowered serum IGFBP5 levels and the IGFBP5/total IGF1 ratio in NAFLD mice, it had no effect on the hepatic expression of IGFBP5, IGF1, and IGF1R. Moreover, exercise increased serum levels of free IGF1 in NAFLD mice, which, when bound to IGF1R, accelerated the phosphorylation levels of hepatic IRS1, Akt, and AMPK α to ameliorate NAFLD. The present study confirmed that exercise activated the effects of IGF1 by reducing the serum IGFBP5/IGF1 ratio, consequently triggering the IRS1/Akt/AMPK pathway to ameliorate HFD-induced NAFLD.

Keywords Exercise, NAFLD, IGFBP5/IGF1 ratio, IRS1, Akt, AMPK

Non-alcoholic fatty liver disease (NAFLD), the most common chronic liver disease, is an escalating public health problem¹, with a prevalence of 25% globally² and approximately 29.2% in China over the last decade³. The worldwide prevalence of NAFLD has continued to grow together with the global epidemic of obesity⁴. Symptoms associated with NAFLD can range from hepatic steatosis to fibrosis/cirrhosis, with some individuals even developing hepatocellular cancer. To date, no gold standard therapy exists for NAFLD, but exercise has been mostly recommended for the preventing and treating health in the United States, Europe, and China^{5–7}. However, the exact mechanisms responsible for the protective and therapeutic effects of exercise against NAFLD have yet to be fully understood.

Insulin-like growth factor-binding proteins (IGFBPs) are a member of the insulin family of proteins. Studies have shown that IGFBP1–6 exhibit high affinity to insulin-like growth factors (IGFs)⁸ and that IGFBP4–6 play important roles as regulators of IGF action⁹. In most circumstances, IGFBPs have a higher affinity for binding with IGFs than with IGF receptors, with 99% of IGFBPs binding with IGFs in the blood⁹. IGFBP5, one of the conserved IGFBPs, it can translocate into the nucleus due to the existence of a nuclear localization sequence¹⁰. IGFBP5 binds to IGFs and modulates its effects on cell proliferation, differentiation, migration, invasion, senescence, apoptosis, and metabolism^{11–14}. The binding affinity between IGFBP5 and IGF1 is higher than that between IGF1 and the IGF1 receptor (IGF1R)⁹. IGFBP5 has been shown to both inhibit and potentiate IGF1 action. Moreover, serum IGFBP5 levels have been correlated with NAFLD. In particular, NAFLD patients

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showed higher serum IGFBP5 levels than did healthy individuals, whereas patients with moderate-to-severe fibrosis displayed significantly higher IGFBP5/total IGF1 levels than did patients with no or mild fibrosis¹⁵. However, the mechanisms underlying these findings have remained unclear.

Exercise can regulate the expression of adipokines and various cytokines in the circulation and in several tissues^{16,17}. Indeed, both acute and chronic exercises have been found to increase the level of IGF1, a member of the IGF family, in the achilles tendon and skeletal muscle^{18,19}; however, other studies have shown that circulating total or free IGF1 either increased, decreased or remained unchanged, demonstrating controversial results^{20–22}. Moreover, exercise showed similar effects on hepatic and circulating total IGF1 expression^{23,24}, whereas both acute and chronic exercise altered circulating IGFBPs. Evidence also shows that IGFBP1–3 and IGFBP-6 all increased after exercise or training^{22,25,26}. In fact, Nindl et al. indicated that exercise increased IGFBP3 and IGFBP4 levels in muscle interstitial fluid²⁰. Another study showed that plasma concentrations of IGF1 and IGFBP3 were significantly decreased after completing an exercise program, with a significant reduction in body weight and body fat in the diet/training group²⁷. Exercise also upregulated the expression of IGFBP7 in muscle satellite cells²⁸ and decreased the expression of IGFBP5 in muscle interstitial fluid²⁰. Nevertheless, the effects of acute and chronic exercise on circulating and hepatic IGFBP5 remains unclear. The current study therefore aimed to investigate the ameliorating effects of regular exercise on NAFLD by determine its impact on circulating and hepatic IGFBP5 levels, as well as the IGFBP5/IGF1 binding ratio in NAFLD mice.

Results

Exercise ameliorates HFD-induced NAFLD characteristics

Hepatic steatosis, inflammation, and fibrosis are considered as the mainly characteristics of NAFLD^{29,30}. HFD-induced NAFLD mice showed increased liver weight, which reduced significantly after regular exercise for 12 weeks (Fig. 1A). Moreover, exercise significantly reduced histological parameters reflecting hepatic steatosis and inflammation, such as the steatosis score, lobular inflammation score, ballooning score, and total NAS (Fig. 1B, C). Hepatic lipid accumulation, total cholesterol levels, triglyceride levels, and collagen accumulation were significantly lower in the HFD + EXE group than in the HFD group (Fig. 1D–G). Exercise downregulated serum alanine aminotransferase and aspartate transaminase levels (Fig. 1H). These results indicated that 12 weeks of regular exercise remarkably alleviated HFD-induced NAFLD.

Exercise relieves HFD-induced insulin resistance

Fasting blood glucose and insulin levels distinctly increased in the HFD-fed mice but both sharply decreased after exercise training (Fig. 2A, B). The HOMA-IR index indicated that HFD-induced insulin resistance was ameliorated in the HFD + EXE mice (Fig. 2C). Exercise training improved tolerance to the glucose challenge (Fig. 2D). The HFD + EXE mice showed reduced blood glucose excursion during ITT compared to HFD mice (Fig. 2E). These results confirmed that exercise protects against insulin resistance.

Exercise decreases serum IGFBP5 levels and the IGFBP5/IGF1 ratio in NAFLD mice

To investigate the mechanism by which exercise ameliorates HFD-induced NAFLD, the related protein profile was analyzed using proteomics. Label-free proteomics was used to detect serum protein profiles in the LFD, HFD, and HFD + EXE groups. The Reactome pathway database contains information on the relationship among molecules in biological pathways and processes, with the presented results being similar to enrichment analysis using Gene Ontology and Kyoto Encyclopedia of Genes and Genomes. Reactome pathway analysis showed that differentially expressed proteins between the three groups were significantly associated with the regulation of IGF transport (Fig. 3A). We then screened for the 10 most significantly differentially expressed proteins using a chord diagram to illustrate the differing protein expression profiles in serum samples. Our results showed that the expression of IGFBP5 in the HFD group was obviously higher than that in the LFD and HFD + EXE groups (Fig. 3B). The expression of IGFBP5 in the HFD group was significantly greater than that in the LFD group using heat maps and enzyme-linked immunosorbent assay (Fig. 3C,D). However, exercise downregulated serum IGFBP5 levels in HFD-induced NAFLD mice (Fig. 3C,D). Although serum total IGF1 levels did not significantly differ between the three groups, free IGF1 levels were obviously higher in the HFD + EXE group than in the HFD group (Fig. 3D). Therefore, these results suggest that exercise decreases the serum IGFBP5/IGF1 ratio in HFD-induced NAFLD mice.

Exercise activated the IRS1/Akt/AMPK pathway by decreasing the serum IGFBP5/IGF1 ratio

We also determined the hepatic expression of IGFBP5, IGF1, and IGF1R in the three groups. Notably, no significant difference in hepatic protein expression levels of IGFBP5, IGF1, and IGF1R were observed between the HFD + EXE and HFD groups (Fig. 4A, B). However, the HFD + EXE group showed greater hepatic phosphorylation levels of IRS1 tyrosine residues, Akt, and AMPK α but lower phosphorylation levels of IRS1 serine residues than did the HFD group (Fig. 4A, B). These data indicated that exercise reduced the serum IGFBP5/total IGF1 ratio to promote the release IGF1, which activated the IRS1/Akt/AMPK signaling pathway.

Discussion

NAFLD is a common liver disease that can become quite harmful when left uncontrolled. Reports have shown that IGFBP5 levels in the liver were correlated with NAFLD⁸. Moreover, many studies have shown that exercise regulates the expression of cytokines in the liver to alleviate NAFLD symptoms, but exercise whether affects the expression of hepatic IGFBP5 has not been reported. The current study found that although NAFLD mice showed decreased hepatic IGFBP5 levels, its expression did not significantly change after 12 weeks of regular exercise. Furthermore, our results showed no obvious change in hepatic IGF1 levels after exercise, which was

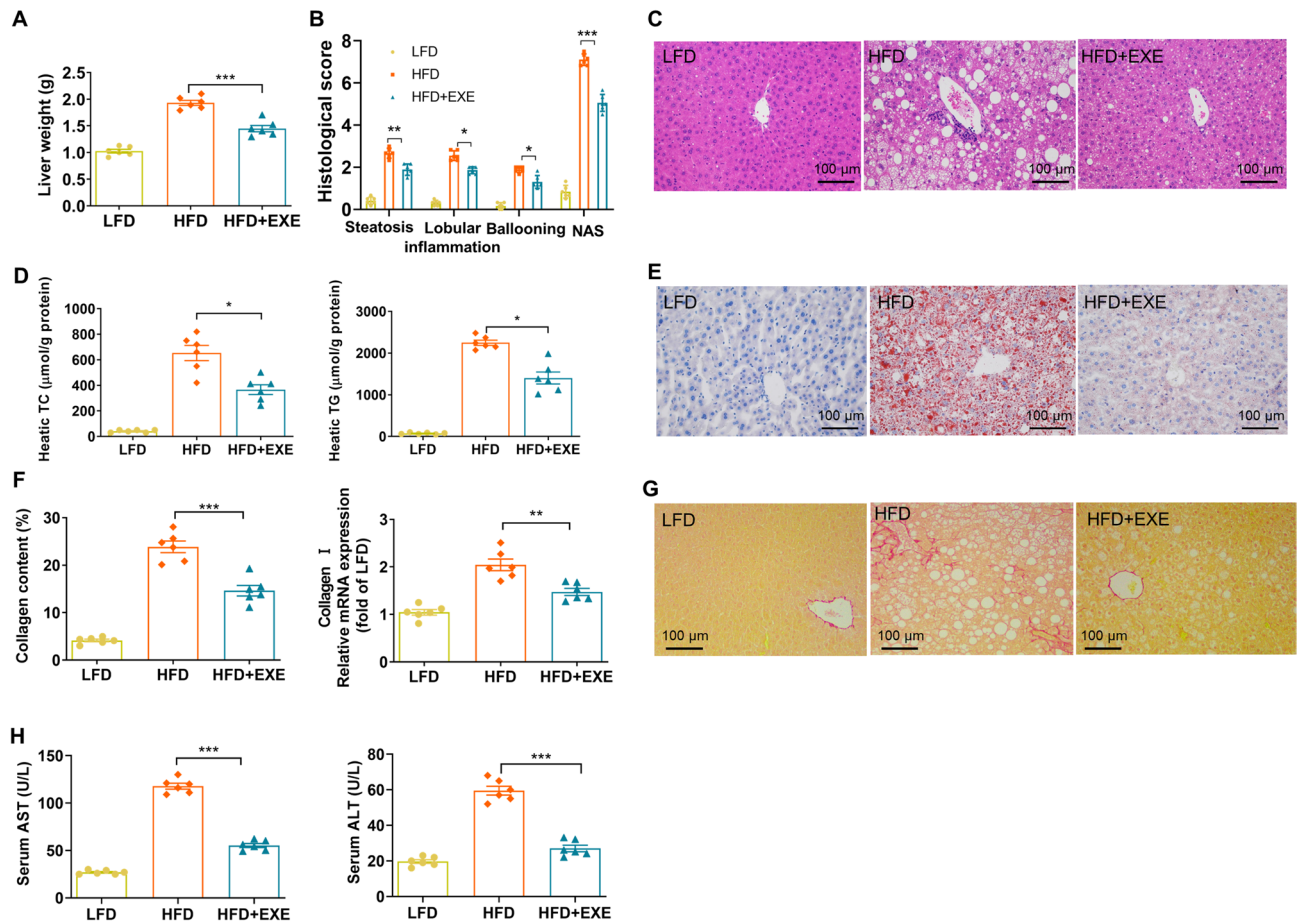


Fig. 1. Exercise reduced HFD-induced liver weight, hepatic steatosis, inflammation, and fibrosis. The liver weight (A) decreased after 12 weeks of treadmill training. Hepatic histological analysis of steatosis, inflammation, ballooning, and NAFLD activity score (NAS) (B). Liver sections stained with H&E ($\times 200$) (C). Hepatic TC levels and TG levels (D). Hepatic lipid accumulation as determined by Oil Red O staining ($\times 200$) (E). Collagen content determined by counting Sirius Red positive areas in six randomly selected fields using Image Pro Plus 6.0 software and hepatic mRNA levels of Collagen I (F). Liver sections stained with Sirius Red ($\times 200$) (G). Serum ALT and AST levels (H). Results are presented as the mean \pm SEM, $n = 6$ per group. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

consistent with the findings of a previous study²³. These results suggested that exercise did not ameliorate NAFLD by increasing or decreasing IGF1 expression in the liver.

The circulating expression of IGF1 has also been associated with the occurrence and development of NAFLD. Indeed, Colak et al. pointed out that serum IGF1 levels in NAFLD patients were higher than those in healthy people¹⁵. The same results were shown in HFD-induced NAFLD mice and in the low-fat diet-induced NAFLD mice. However, the same study showed that serum IGF1 levels in NAFLD mice decreased significantly after 12 weeks of regular exercise and that the serum total IGF1 levels did not differ between NAFLD mice with and without exercise. It indicated that exercise decreased the IGF1/total IGF1 by reduced IGF1 expression rather than increased IGF1 expression in serum. Although the previous study have indicated that IGF1/total IGF1 is associated with NAFLD, the mechanism remains unclear. How exercise reduced IGF1/total IGF1 to alleviate NAFLD? We carried out experimental research on the molecular mechanism to answer this question.

Studies have reported that serum IGF1 levels were correlated with NAFLD and that its levels were decreased in NAFLD individuals^{31,32}. Another study found that low serum IGF1 levels can be a predictor of advanced hepatic fibrosis³³. IGF1 has been found to have a high affinity with IGF1 and can inhibit and potentiate IGF1 action in the circulation⁹. If the level of IGF1 is lower in the circulation, IGF1 combined with it will decrease, which useful for activating the signaling pathway mediated by IGF1. Therefore, a decline in the serum IGF1/IGF1 ratio can increase free IGF1 levels and promote its activation. Some studies indicated that IGF1 levels increased or decreased after exercise in circulating, muscle interstitial fluid and muscle satellite cells^{20,23,24,26}. It is reported that exercise downregulated the expression of IGF1 in muscle interstitial fluid¹⁸. However, the effect on hepatic IGF1 by exercise has not been reported yet. We discovered that serum IGF1 decreased and hepatic IGF1 had no significant change. The current study also found that the IGF1/total IGF1 ratio was lower and the free IGF1 levels were higher after the exercise intervention. It is reported that long-term exercise induced a significant increase of plasma volume in healthy untrained individuals, but the effect of long-term

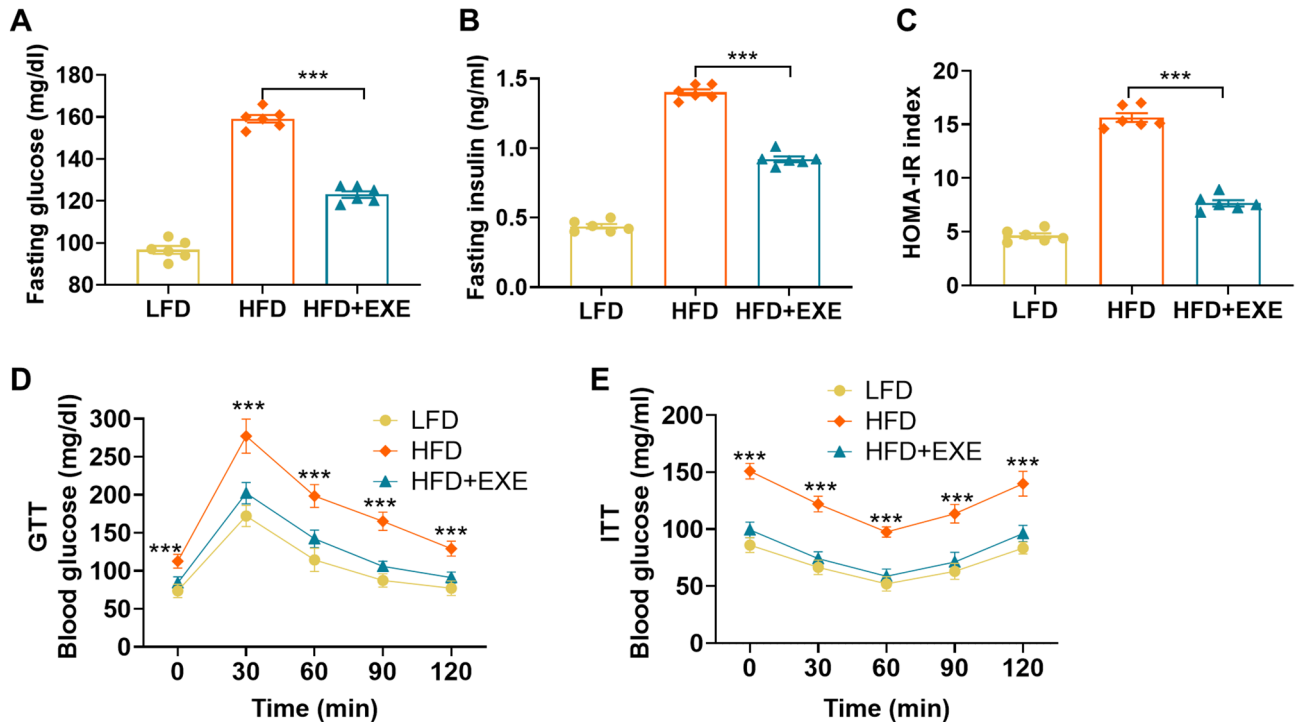


Fig. 2. Exercise relieves HFD-induced insulin resistance. Fasting blood glucose (A), serum insulin levels (B) and the HOMA-IR index (C). Glucose tolerance test (GTT) (D) and insulin tolerance test (ITT) (E). Results are presented as the mean \pm SEM, $n = 6$ per group. *** $P < 0.001$ (HFD vs. HFD + EXE).

exercise on plasma volume in trained individuals is no consensus concerning³⁴. The serum volume of HFD mice and HFD + EXE mice was no significant different in this study. These results indicated that exercise reduced the serum IGFBP5/IGF1 ratio to activate the action of IGF1 rather than exercise regulated the hepatic IGFBP5 levels, resulting in the amelioration of NAFLD.

Insulin resistance is vital for the development of NAFLD³⁵ and exercise can decline insulin resistance by reducing adipokines and proteins^{16,17,36}. IRS1 and protein kinase B (Akt) are essential factors for the insulin signaling pathway³⁷. One study showed that under NAFLD condition, tyrosine phosphorylation levels of IRS1 and phosphorylation levels of Akt in the livers were weakened, whereas serine phosphorylation levels of IRS1 were enhanced³⁸. Our study showed that exercise promoted IGF1 binding to its receptor by reducing the serum IGFBP5/IGF1 ratio, which weakened IRS1 serine phosphorylation and enhanced the phosphorylation of IRS1 tyrosine residues and Akt. Surprisingly, exercise also increased AMPK α phosphorylation levels in the liver. Hepatic and adipose AMPK activation can increase insulin sensitivity³⁹, whereas its phosphorylation can suppress de novo lipogenesis in NAFLD^{40,41}.

In Conclusion, although exercise did not affect IGFBP5 expression in the liver, it activated the effects of IGF1 by reducing the serum IGFBP5/IGF1 ratio in NAFLD mice (Fig. 5). The release of IGF1 then triggered IRS1/Akt/AMPK signaling pathway to ameliorate HFD-induced NAFLD (Fig. 5).

Materials and methods

Animal model

Five-week-old male C57BL/6J mice were purchased from the Experimental Animal Center of Southern Medical University (Guangzhou, China) and acclimated for 1 week. The mice were housed on a 12-h light–dark cycle at 22°C–24°C and were provided free access to food and water. All animal care and laboratory experimental procedures were conducted in accordance with the Chinese Guidelines for Animal Welfare and Experimental Protocols and were approved by the Animal Experiment Administration Committee of Guangzhou Sport University (2022DWLL-28). All mice were randomly divided into a low-fat diet control group (LFD, $n = 8$), a high-fat diet group (HFD, $n = 8$), and a HFD plus exercise group (HFD + EXE, $n = 8$). The LFD mice were fed a diet of 10% kcal from fat (D12450J, Research Diets Inc.) throughout the experimental period. The other two groups received a HFD containing 60% kcal from fat (D12492, Research Diets Inc.) for 24 weeks. After 12 weeks of HFD feeding, the HFD + EXE group mice were subjected to training on a treadmill at a 0% grade for 12 weeks. A day after the phase of final training, the serum and liver samples were collected from sacrificed mice under anesthesia (intraperitoneal-injected sodium pentobarbital 50 μ g/g).

Exercise protocol

The exercise mice were trained on a treadmill at 0% grade 5 days per week for 12 weeks. The first week is an adaptation period, a 5 min warm-up period at 6 m/min, a 20 min of the main exercise at 10 m/min, and a 5 min

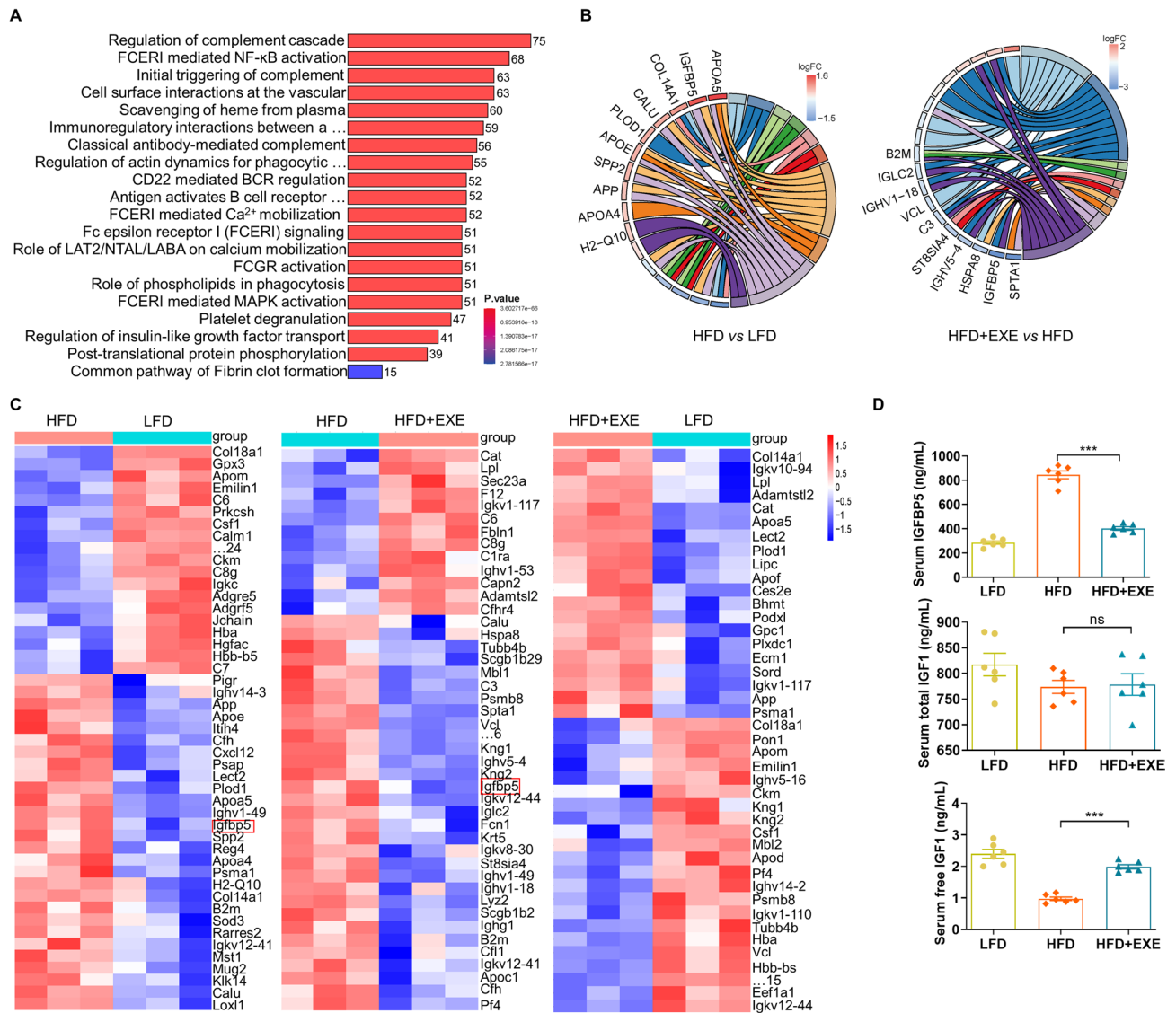


Fig. 3. Exercise reduced HFD-induced serum IGF1/IGFBP5 ratio. All identified proteins were classified according to the top 20 gene ontology ratios sorted by enrichment degree [$-\log_{10}(p\text{-value})$] (A). Chord diagram of top 10 differentially expressed proteins among the LFD, HFD and HFD + EXE groups (red represents up-regulation, blue represents down-regulation) (B). Heat map of differentially expressed proteins among the LFD, HFD and HFD + EXE groups (red represents up-regulation, blue represents down-regulation) (C). ELSA results of serum IGF1 and free IGF1 levels (D). Results are presented as the mean \pm SEM, $n = 3$ or 6 per group. $^{ns}P > 0.05$ and $^{***}P < 0.001$.

of cool down at 6 m/min were performed. The mice performed 5 min warm up session at 6 m/min, a 50 min of the main training at 12 m/min, and a 5 min of cool down at 6 m/min from the 2nd to the final week.

Blood analysis

The serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were determined using commercial kits (Jiancheng Bioengineering Institute, Nanjing, China).

Glucose tolerance test (GTT) and insulin tolerance test (ITT)

A GTT was carried out after 11 weeks of diet and exercise intervention. The levels of blood glucose were detected after overnight fasting using a glucometer (Accu-Chek, Roche). Thereafter, mice received an intraperitoneal injection of glucose at a dose of 1 mg/g body weight, after which blood collection was from the tail vein at 0, 30, 60, 90, 120 min after injection, respectively.

An ITT was performed in the previous week of final diet and exercise intervention. The mice were performed an intraperitoneal injection of insulin (Sigma) at a dose of 0.75 IU/kg body weight after a 3-h fast. Blood glucose levels were determined from tail vein at 0, 30, 60, 90, and 120 min after injection using a glucometer (Accu-Chek, Roche).

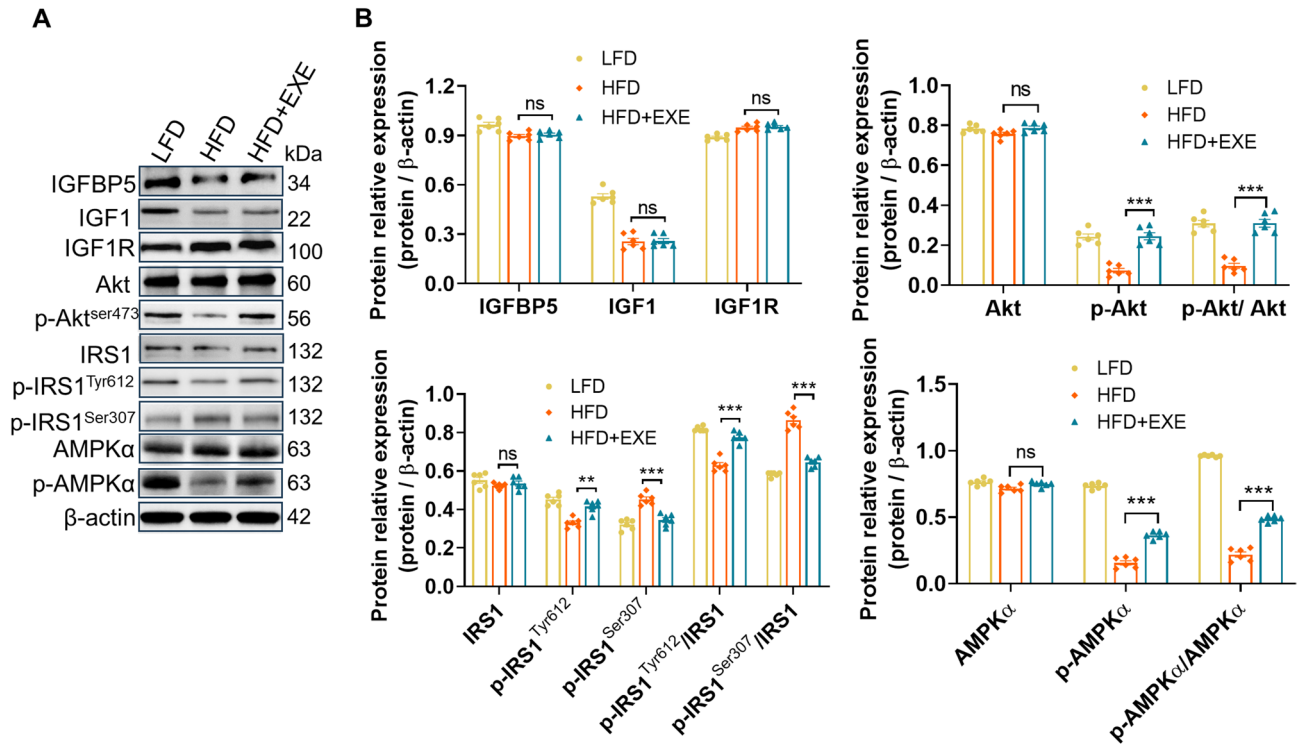


Fig. 4. Exercise activated IRS1/Akt/AMPK pathway due to reduce serum IGFBP5/total IGF1 ratio. (A). Protein levels of IGFBP5, IGF1, IGF1R, IRS1, p-IRS1 (Ser307), p-IRS1 (Tyr612), Akt, p-Akt (Ser473), AMPKα and p-AMPKα (Thr183/172) in mouse livers were assayed by western blot. (B). Protein expression results are presented as the mean ± SEM, *n* = 6 per group. *ns* *P* > 0.05, **** *P* < 0.01 and ***** *P* < 0.001.

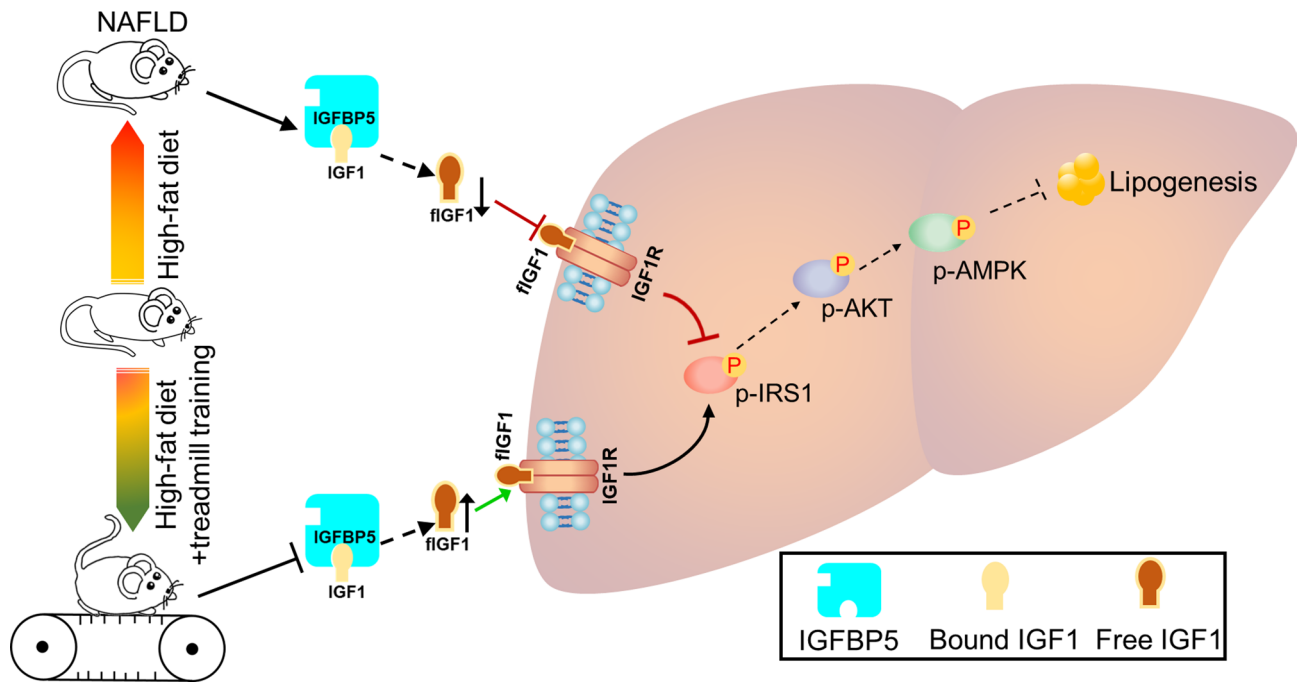


Fig. 5. Exercise activated the effects of IGF1 by reducing the serum IGFBP5/IGF1 ratio, consequently triggering the IRS1/Akt/AMPK signaling pathway to ameliorate HFD-induced NAFLD.

Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')
Collagen I	GCCCGAACCCCAAGGAAGAAGC	CTGGGAGGCCTCGGTGACATTAG
GAPDH	CCTCGTCCCGTAGACAAAATG	TGAGGTCAATGAAGGGTCTGT

Table 1. Primer sequences used for qRT-PCR.

Homeostatic model assessment for insulin resistance (HOMA-IR) calculation

HOMA-IR was calculated to evaluate the insulin resistance. The formula is as follows: $\text{HOMA-IR} = \text{fasting glucose (mmol/L)} \times \text{fasting insulin (mIU/L)} / 22.5^{42}$.

Hepatic triglyceride and total cholesterol analysis

Hepatic TG and TC levels were measured using commercial kits (Jiancheng Bioengineering Institute, Nanjing, China), according to the manufacturer's instructions.

Histological analysis of the liver

Fresh liver tissues were fixed with 4% paraformaldehyde solution for 24 h, embedded in paraffin, and sliced into 4-mm sections for hematoxylin–eosin (H&E) and Sirius Red staining. The NAFLD activity score (NAS) was then calculated according to the guidance supplied by the Pathology Committee of the NASH Clinical Research Network⁴³: steatosis (<5% = 0; 5–33% = 1; 33–66% = 2; >66% = 3), lobular inflammation (none = 0; <2 foci = 1; 2–4 foci = 2; >4 foci = 3), and hepatocellular ballooning (none = 0; few = 1; prominent = 2). All features of per sample were scored in a blinded manner based on six fields of view. Individual scores for each field of view were summed to estimate the NAS for each mouse.

Serum proteomic analyses

Label-free mass spectrometry technology, coupled with liquid chromatography with tandem mass spectrometry, was used to quantitatively analyze proteins and identify those differentially expressed. All such analyses were performed by Metabo-Profile Biotechnology (Shanghai) Co., Ltd., China. Results were analyzed using FragPipe software, whereas protein data were screened from the UniProt database.

ELISA assays

The serum levels of total IGF1 (Mediagnost, Reutlingen, Germany), free IGF1 (AnshLabs, Webster, TX, USA) and IGFBP5 (Raybiotech, Norcross, Georgia, USA) were measured following the manufacturers' instructions. Absorbance was read by spectrophotometry (BioTek, Winooski, USA).

Quantitative real-time PCR

Total RNA was extracted from frozen liver using RNeasy Plus Mini Kit (Qiagen) following the manufacturer's instructions. The primer sequences are detailed in Table 1. Expression levels were normalized to those of the housekeeping gene GAPDH.

Western blot analysis

After extracting the protein from experimental animal livers, total protein concentrations were measured using a BCA protein assay kit (Thermo Fisher Scientific). Equal amounts of total protein were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. These membranes were then blocked and incubated with primary antibodies against IGF1 (Abcam, ab254324, Cambridge, MA, USA), IGF1 (Abcam, ab223567, Cambridge, MA, USA), IGF1R (Abcam, ab182408, Cambridge, MA, USA), IRS1 (Proteintech, 17509, China), phospho-IRS1 (Tyr612) (Thermo Fisher, 44816G, Waltham, MA, USA), phospho-IRS1 (Ser307) (Bioss, bs3200R, China), Akt (Servicebio, GB15689, China), phospho-Akt (Ser473) (ImmunoWay, YP0006, Plano, TX, USA), AMPK α (ImmunoWay, YT0216, Plano, TX, USA), phospho-AMPK α (Thr183/172) (ImmunoWay, YP0575, Plano, TX, USA), and β -actin (Servicebio, GB15003, China). The membranes were incubated for 1 h with the following secondary antibody: goat anti-rabbit IgG-HRP (Servicebio, GB23303, China). The immunoblot signals were quantified using Quantity One software after signal detection.

Statistical analysis

All data were presented expressed as mean \pm standard error of the mean (mean \pm SEM). One-way analysis of variance followed by Tukey's test for three groups comparisons. A *t*-test was used for two groups comparisons in the analysis of protein profile. All analyses were performed using GraphPad Prism (version 8.0.2), with a *P* value less than 0.05 indicating statistical significance.

Data availability

The datasets used in the analyses described in this study are available from the corresponding author on reasonable request.

Received: 13 March 2025; Accepted: 17 June 2025

Published online: 02 July 2025

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Acknowledgements

The authors thank all the laboratory members for their help (Haohui Lai, Jianhua Wang, Wenbi He, Huan Wang, Gaoyuan Yang and Yu Wang).

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Funding

This work was supported by the Department of Education of Guangdong Province, China. (2023ZDZX2034) (to Y.Y.), and the Science and Technology Plan Project of Liwan District of Guangzhou, China (to L.Y.).

Declarations

Competing interests

The authors declare no competing interests.

Ethics statement

All animal experiments in this study were performed in accordance with the ARRIVE guidelines 2.0, and the Institutional Animal Care and Use Committee of Guangzhou Sport University approved all procedures performed on animals.

Additional information

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1038/s41598-025-07857-y>.

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