

Heterophyllin B enhances the benefits of intermittent fasting in the treatment of metabolic dysfunction-associated steatotic liver disease via activation of GLP-1R

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Received March 14, 2025; Accepted July 2, 2025

DOI: 10.3892/ijmm.2025.5641

Abstract. Intermittent fasting (IF) has shown particularly promising short-term effects in improving metabolic dysfunction-associated steatotic liver disease (MASLD), although its long-term efficacy remains unclear. Heterophyllin B (HP-B), a cyclopeptide compound derived from *Pseudostellaria heterophylla*, is known for its potent anti-inflammatory and hypoglycemic properties. However, studies investigating the potential role of HP-B in the management of MASLD are lacking. *In vitro*, an OA/PA-induced lipid accumulation model was established using HepG2/Huh-7 cells. The therapeutic effects of HP-B and fasting-mimicking conditions were evaluated through Cell Counting Kit-8 assay, Oil Red O staining, reverse transcription-quantitative PCR, and western blot analysis. For *in vivo* studies, C57BL/6J mice were fed a high-fat diet and treated with HP-B, IF, or their combination. Mechanistic validation was performed via adenovirus-mediated GLP-1R knockdown. The present study aimed to explore whether HP-B can serve as an adjunctive supplement to enhance the benefits of IF in the treatment of MASLD. HepG2 and Huh-7 liver cancer cells treated with oleic acid/palmitic acid (OA/PA) presented significant lipid accumulation, which was attenuated by HP-B treatment and fasting. The combination treatment markedly reduced lipid levels and oxidative stress, as well as restored the mitochondrial membrane potential, with a synergistic effect over treatment alone. In addition, the combination of HP-B and fasting upregulated glucagon-like peptide-1 receptor (GLP-1R) and peroxisome proliferator-activated

receptor gamma coactivator 1-alpha expression, reversing the OA/PA-induced decline. In high-fat diet-fed mice, the combination treatment reduced hepatic lipid accumulation, decreased liver weight, decreased mouse body weight, and improved biochemical indices of liver function. The beneficial effects of HP-B and fasting were reversed after silencing GLP-1R with small interfering RNA or Ad-GLP-1R, emphasizing the critical role of GLP-1R in mediating these protective effects. In conclusion, the synergistic effects of HP-B and fasting on improving lipid metabolism and mitochondrial function are mediated primarily through the regulation of GLP-1R, making it a promising therapeutic target for the treatment of MASLD and other lipid metabolism-related disorders.

Introduction

Metabolic dysfunction-associated steatotic liver disease (MASLD) has emerged as a global public health concern and is characterized by the accumulation of fat in the liver in the context of metabolic dysfunction. The pathophysiology of MASLD is complex, with oxidative stress and insulin resistance (IR) playing central roles in its development and progression (1). The increasing prevalence of MASLD is closely linked to increasing rates of obesity, type 2 diabetes, and other metabolic disorders, making it a significant health issue worldwide. In the search for effective management strategies, intermittent fasting (IF), also known as time-restricted feeding, has garnered attention as a potential therapeutic approach. IF involves alternating between periods of normal eating and fasting, with variations such as alternate-day fasting and periodic fasting (2). The relatively simple structure of IF has contributed to its widespread popularity as a dietary regimen among the general population. Studies have suggested that IF may be beneficial in alleviating liver fat accumulation by increasing insulin sensitivity, reducing fat deposition, and modulating inflammatory responses, which are crucial factors in the pathogenesis of MASLD (3). However, while IF shows promise in managing MASLD, it has some challenges. Certain individuals, particularly those with hypoglycemia, diabetes, or gastrointestinal disorders, may face health risks associated with IF (4). Furthermore, although short-term IF has demonstrated

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Key words: heterophyllin B, intermittent fasting, metabolic dysfunction-associated steatotic liver disease, glucagon-like peptide-1 receptor, peroxisome proliferator-activated receptor gamma coactivator 1-alpha

beneficial effects on liver fat reduction and liver enzyme levels in MASLD, the long-term impact remains inadequately studied. Some research has indicated that the sustainability of IF may be limited, especially after periods of refeeding, when hepatic steatosis may relapse (5). Therefore, while IF presents a potential strategy for MASLD management, the effects of IF are likely to vary depending on individual health conditions and dietary habits.

Studies have shown that peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 α) plays a critical role in the onset and progression of IR (6). In the liver, PGC-1 α activates key enzymes involved in gluconeogenesis, promoting hepatic glucose production, which results in increased hepatic glucose output (7). Unrestrained hepatic glucose output is a hallmark of IR. In addition, mitochondrial dysfunction is implicated in the development of MASH (8). Mitochondrial ultrastructural damage has been observed in the livers of patients with non-alcoholic steatohepatitis, with a decrease in the quantity and activity of respiratory chain complexes and impaired ATP synthesis (8). PGC-1 α influences mitochondrial function by enhancing mitochondrial respiration, a process in which PGC-1 α strongly induces the mRNA expression of nuclear respiratory factor (NRF)-1 and NRF-2 α (9). NRF-1 and NRF-2 α are key regulators of genes involved in the mitochondrial respiratory chain, including cytochrome c oxidase IV (COX IV), β -ATP synthase, and mitochondrial transcription factor A (mtTFA) (9). Furthermore, dysregulated lipid metabolism generates large amounts of reactive oxygen species (ROS), disturbing the dynamic balance between pro-oxidants and antioxidants, which leads to oxidative stress and lipid peroxidation (7,10,11). PGC-1 α is involved in hepatic lipid metabolism and plays a crucial role in reducing hepatic fat accumulation (12). Recent studies have highlighted the important role of the PPAR α nuclear receptor transcription factor in the transcriptional regulation of intracellular lipid metabolism (12,13). PPAR α controls the transcriptional activity of genes such as long-chain acyl-CoA dehydrogenase, medium-chain acyl-CoA dehydrogenase and carnitine palmitoyltransferase-1. PGC-1 α synergistically stimulates PPAR α to increase the transcriptional activity of fatty acid oxidases (13). Overall, PGC-1 α plays a protective role in the mechanisms currently recognized in MASLD, such as IR, mitochondrial damage, and lipid metabolism dysregulation, by increasing insulin sensitivity, promoting mitochondrial respiration, and facilitating fatty acid β -oxidation, thus alleviating hepatic triglyceride accumulation.

Heterophyllin B (HP-B) is a cyclopeptide compound derived from plants that has shown significant progress in research on blood glucose regulation and anti-inflammatory effects in recent years (14-16). HP-B interacts with the glucagon-like peptide-1 receptor (GLP-1R), mimicking the actions of GLP-1 analogs, promoting insulin secretion, and improving insulin sensitivity, thereby modulating metabolic disturbances (14). These actions are particularly relevant in the context of MASLD, the pathogenesis of which is closely linked to IR, fat deposition, oxidative stress and chronic inflammation (17). Given the potential of HP-B to regulate metabolic disturbances and reduce oxidative stress, it was hypothesized that the combination of HP-B

with IF may have a synergistic effect on MASLD. More importantly, the integration of a natural GLP-1R activator with a dietary intervention strategy may provide a more effective and tolerable therapeutic option for patients with MASLD, particularly those unable to adhere to strict fasting protocols. By elucidating the molecular mechanisms underlying this combinatorial approach, the present findings may contribute to the optimization of MASLD treatment strategies and advance the development of personalized metabolic therapies.

Materials and methods

Cell culture. HepG2 and Huh-7 liver cancer cell lines were purchased from Procell Life Science & Technology Co., Ltd. STR profiling was used for authentication. The cells were cultured in DMEM (MedChemExpress) supplemented with 10% fetal bovine serum (FBS; HyClone; Cytiva) and 1% penicillin-streptomycin (Beijing Solarbio Science & Technology Co., Ltd.) in an incubator at 37°C with 5% CO₂. The cells were divided into the following groups: Control (Con) group, in which the cells were treated with vehicle (0.1% DMSO in PBS) for 24 h; OA/PA model group, in which HepG2 and Huh-7 cells were treated with 750 μ M oleic acid + 750 μ M palmitic acid (2:1 ratio) for 24 h (18), followed by treatment with vehicle (0.1% DMSO in PBS) for 24 h; OA/PA + HP-B group, in which the cells were treated with OA/PA for 24 h, followed by treatment with 50 μ M HP-B (prepared from 10 mM DMSO stock diluted in PBS) for 24 h; OA/PA + Fasting group, in which the cells were treated with OA/PA for 24 h, followed by culture in low-glucose DMEM + 2% FBS + vehicle (0.1% DMSO) for 24 h; and OA/PA + Fasting + HP-B group, in which the cells were treated with OA/PA for 24 h, followed by cultured in fasting medium (low-glucose DMEM + 2% FBS) containing 50 μ M HP-B for 24 h.

Cell counting kit-8 (CCK-8) assay. HepG2 and Huh-7 liver cancer cells were seeded at a density of 5x10³ cells/well in 96-well plates and allowed to adhere overnight at 37°C. Following treatment with 5, 10, 25, 50, 75, 100, or 200 μ M HP-B for 24 h, cell viability was then determined according to the instructions of the CCK-8 assay kit (Beijing Solarbio Science & Technology Co., Ltd.). In brief, 10 μ l CCK-8 solution was added to each well containing 100 μ l culture medium and incubated at 37°C for 2 h in a humidified incubator with 5% CO₂. Optical density was measured at 450 nm using a microplate reader (Thermo Fisher Scientific, Inc.).

Oil Red O staining. Following treatment of the HepG2 and Huh-7 liver cancer cells, the media was removed, and the cells were washed twice with PBS. The cells in each well were fixed for 10 min at room temperature with 4% paraformaldehyde (Beijing Solarbio Science & Technology Co., Ltd.). The cells were subsequently stained with 0.5% Oil Red O solution at room temperature for 10 min. After staining, the excess dye was removed by washing with 60% isopropanol, followed by three washes with distilled water (5 min each wash) to prevent non-specific binding of the dye. Under an inverted microscope (Olympus Corporation), the cells were examined, and images of representative fields were acquired.

Determination of total cholesterol (TC) and triglyceride (TG) levels. Following the treatment of HepG2 and Huh-7 liver cancer cells, the supernatants were collected. For the liver tissue samples, a homogenizer was used to homogenize the liver tissue. The samples were subsequently centrifuged for 10 min at 4°C and 12,000 x g, after which the supernatants were collected. The TG and TC levels were measured by TG (cat. no. A110-1-1) and mouse total cholesterol ELISA kit (cat. no. A111-1-1; both from Nanjing Jiancheng Bioengineering Institute) according to the manufacturer's protocols.

Reverse transcription-quantitative PCR (RT-qPCR). Following the manufacturer's instructions, TRIzol reagent (Beijing Solarbio Science & Technology Co., Ltd.) was used to extract total RNA from HepG2 and Huh-7 human liver cancer cells. The concentration and purity (2.0 > OD_{260/280} > 1.8) of the RNA were determined using a Multiskan SkyHigh nanodrop (Thermo Fisher Scientific, Inc.). Following the instructions supplied by the PrimeScript RT reagent kit (Takara Bio, Inc.), 1 µg of total RNA was used for reverse transcription to synthesize cDNA. qPCR was then performed using SYBR Green qPCR Master Mix (Takara Bio, Inc.). The qPCR program was as follows: Pre-denaturation at 95°C for 5 min; and 35 cycles of denaturation at 95°C for 10 sec and annealing/extension at 60°C for 30 sec. Relative quantification analysis was performed using the 2^{-ΔΔC_q} method with GAPDH used as the reference gene, and the relative expression levels of the target genes were calculated and expressed as 2^{-ΔΔC_q} (19). The primers used in the present study were listed as follows: SREBP1 forward, 5'-TGCTTAGCCTCCTGACCTGA-3' and reverse, 5'-GAGGCCCTAAGGGTTGACAC-3'; FAS forward, 5'-CAGTGTACACGTCTGGACCC-3' and reverse, 5'-AAT TGTGGGAGGCTGAGAGC-3'; CD36 forward, 5'-ACATG CTAGCCACTGATCATTTT-3' and reverse, 5'-ACAACCTTG GCACAAGTGCTTT-3'; GLP-1R forward, 5'-TTGGGTGGT GCAACCTTTCT-3' and reverse, 5'-GATGCTGGAGTTGGT CTGCT; PGC1α forward, 5'-ATTGCCTTCATGCCGTGG TA-3' and reverse, 5'-GCAAGGGCTCAACTAATCGC-3'; and GAPDH forward, 5'-AATGGGCAGCCGTTAGGAAA-3' and reverse, 5'-GCGCCCAATACGACCAAATC-3'.

Detection of ROS levels. HepG2 and Huh-7 liver cancer cells were plated at a density of 3x10⁵ cells/well in 6-well plates. After overnight incubation, the medium was discarded, and the cells were gently washed twice with PBS. Then, 2 ml of 10 µM DCFH-DA (Beijing Solarbio Science & Technology Co., Ltd.) working solution was added to each well and incubated at 37°C in the dark for 30 min. After incubation, the DCFH-DA working solution was discarded, and the cells were gently washed twice with PBS. The medium was replaced with serum-free medium. Fluorescent images were acquired using a fluorescence microscope (Olympus Corporation).

Detection of mitochondrial ROS (mitoROS) levels. HepG2 and Huh-7 liver cancer cells were seeded in 6-well plates at a density of 3x10⁵ cells/well. Following an overnight incubation period, the medium was discarded, and the cells were gently washed twice with PBS. Then, 2 ml of 5 µM MitoSOX Red (Beijing Solarbio Science & Technology Co., Ltd.) working solution was added to each well, followed by incubation at

37°C in the dark for 30 min. Following incubation, the cells were gently washed twice with PBS, and the medium was replaced with 2 ml of serum-free media after the MitoSOX Red working solution was removed. Fluorescent images were acquired using a fluorescence microscope (Olympus Corporation).

Detection of the mitochondrial membrane potential (MMP). HepG2 and Huh-7 liver cancer cells were seeded in 6-well plates at a density of 3x10⁵ cells/well. Following an overnight incubation period, the medium was discarded, and the cells were gently washed twice with PBS. Next, 2 ml of 5 µg/ml JC-1 working solution (Beijing Solarbio Science & Technology Co., Ltd.) was added to each well, followed by incubation for 20 min at 37°C in the dark. After incubation, the JC-1 working solution was discarded, and the medium was replaced with serum-free medium. The fluorescent JC-1 signals in the cells were detected using a fluorescence microscope (Olympus Corporation).

Western blot analysis. The HepG2 and Huh-7 liver cancer cells or liver tissues were collected and lysed using a RIPA lysis solution (Beijing Solarbio Science & Technology Co., Ltd.) on ice for 30 min. The samples were subsequently centrifuged for 10 min at 4°C and 12,000 x g. The protein concentration was measured using a BCA kit (Beijing Solarbio Science & Technology Co., Ltd.). Protein samples (20 µg/lane) were separated by 12% SDS-PAGE. After being transferred, the PVDF membrane was blocked for 1 h at room temperature in 5% non-fat milk. After blocking, the membrane was washed three times with TBST (0.1% Tween-20, 5 min each wash). The membrane was incubated overnight at 4°C with specific primary antibodies: anti-PGC1α (cat. no. ab176328; 1:1,000), anti-GLP1R (cat. no. ab218532; 1:1,000) and anti-GAPDH (cat. no. ab8245; 1:5,000; all purchased from Abcam). The membrane was then incubated at room temperature for 1 h in antibody dilution buffer containing HRP-conjugated secondary antibodies (SE205; cat. no. K21001M-HRP; Beijing Solarbio Science & Technology Co., Ltd.). The membrane was then washed three times with TBST (10 min each wash). The proteins in the membrane were detected via ECL reagent (Beijing Solarbio Science & Technology Co., Ltd.). ImageJ version 2 (National Institutes of Health) was used to assess the band intensity, and GAPDH was used as a loading control.

Establishment of animal model. In this experiment, a total of 45 8-week-old male C57BL/6J mice [weight, 20-22 g; SiPeiFu, (Beijing) Biotechnology Co., Ltd.] were randomly assigned to 9 experimental groups, with 5 mice per group. The first phase of the experiment included the following groups: i) control group (Con), in which the mice were fed a standard chow diet with daily oral vehicle (10% DMSO/corn oil, 200 µl) for 8 weeks; ii) HFD group, in which the mice were fed a high-fat diet (HFD; 45% fat, D12451, Research Diets, Inc.) for 8 weeks to induce MASLD and then subsequently fed a daily oral vehicle (10% DMSO/corn oil, 200 µl) for 8 weeks; iii) HFD + HP-B group, in which the mice were fed a HFD for 8 weeks, followed by oral administration of HP-B (20 mg/kg/day; total volume of 200 µl) for 8 weeks (20); iv) HFD + IF group, in which the mice were fed a HFD for 8 weeks, followed by IF

for 8 weeks (two 24-h fasts/week: Wed 9AM→Thu 9AM and Fri 9AM→Sat 9AM) with daily oral administration of vehicle (10% DMSO/corn oil, 200 μ l); and v) HFD + IF + HP-B group, in which the mice were fed a HFD for 8 weeks, followed by combined HP-B administration (20 mg/kg/day) and IF for 8 weeks.

In the second phase (initiated after 8 weeks of HFD induction), the mice received a single tail vein injection of adenovirus (Day 0), followed by 8 weeks of intervention. To ensure sustained GLP-1R knockdown, Ad-sh-GLP-1R [5'-AGTGTCTGAAGCCAACAAGGA-3', designed against the mouse GLP-1R mRNA (NM_021332)] and an adenoviral vector expressing a non-targeting scrambled shRNA sequence (Ad-NC, 5'-TTCTCCGAACGTGTCACGT-3') were readministered at Week 4. The second phases consisted of the following groups: i) HFD + Ad-NC group, in which the mice were injected with Ad-NC (100 μ l, 10^{11} pfu/ml) on Day 0 and Week 4, followed by daily oral administration of vehicle (10% DMSO/corn oil, 200 μ l) for 8 weeks; ii) HFD + IF + HP-B + Ad-NC group, in which the mice were injected with Ad-NC on Day 0 and Week 4, followed by continued IF + HP-B treatment (20 mg/kg/day) for 8 weeks; iii) HFD + sh-GLP-1R group, in which the mice were injected with Ad-sh-GLP-1R (100 μ l, 10^{11} pfu/ml) on Day 0 and Week 4, followed by daily oral administration of vehicle for 8 weeks; and iv) HFD + IF + HP-B + Ad-GLP-1R group, in which the mice were injected with Ad-shPGC1 α on Day 0 and Week 4, followed by IF + HP-B treatment for 8 weeks.

The mice were housed under standard laboratory conditions with free access to water at a temperature of 24°C and a humidity of 45%, with a 12/12-h light/dark cycle. Anesthesia was induced with 3–5% isoflurane, and deep anesthesia was confirmed by the absence of a response to pain stimuli; 2% isoflurane was used to maintain anesthesia during the procedures. At the end of the experimental period, all mice were euthanized under deep anesthesia with isoflurane (5% in oxygen), followed by cervical dislocation to ensure death. Death was confirmed by the cessation of heartbeat and respiratory movement, as well as the loss of reflexes.

In the first phase of the experiment, all the mice were sacrificed at the end of the 8-week HFD treatment, and blood and liver tissue samples were collected for analysis. For the IF group all the mice were sacrificed within 48 h after the last fasting cycle. The mice from all the groups were sacrificed at the end of the experiment (after 8 weeks). Blood samples were collected from the heart, and liver samples were obtained. In addition, liver weights were recorded. The body weights were measured regularly, and the liver index (liver weight/body weight) was calculated. Additionally, liver histology, serum marker assessment of liver function, and gene/protein expression analysis were performed to assess the effects of the treatments. The present study was conducted in strict accordance with ethical guidelines for animal research and all applicable national regulations. The experimental protocol was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Beijing University of Aeronautics and Astronautics (approval no. 2023-KY-064-1; Beijing, China). All procedures were designed to minimize animal suffering, and humane care was provided throughout the study. In addition, all the

animal experiments complied with the ARRIVE guidelines and the NIH Guide for the Care and Use of Laboratory Animals.

Insulin tolerance test (ITT). ITT was conducted by first administering an intraperitoneal (IP) injection of insulin at a dose of 1 U/kg body weight to induce insulin action. Blood samples were then collected from the tail tip at 0, 15-, 30-, 45-, 60- and 90-min post-injection to measure blood glucose levels using a portable glucometer.

Glucose tolerance test (GTT). Prior to the GTT, the mice were fasted for 6 h (with free access to water) to standardize the baseline blood glucose levels. The baseline blood glucose value was measured at 0 min (pre-injection) via tail-tip blood sampling. A sterile glucose solution (20% w/v) was then administered via IP injection at a dose of 2 g/kg body weight. Blood samples were subsequently collected from the tail tip at 15-, 30-, 45, 60- and 120-min post-glucose loading, and blood glucose levels were analyzed at each time point using a portable glucometer.

H&E staining. The liver tissues were collected and fixed for 24 h in a 4% paraformaldehyde solution (Beijing Solarbio Science & Technology Co., Ltd.). The fixed liver tissues were then dehydrated, embedded in paraffin and sectioned into 6- μ m thick sections. The sections were sequentially immersed in xylene and a gradient of ethanol solutions for deparaffinization and rehydration. The sections were then washed with tap water after being stained for 5 min in a solution of hematoxylin (Beijing Solarbio Science & Technology Co., Ltd.). The sections were then stained with an eosin solution (Beijing Solarbio Science & Technology Co., Ltd.) for 1 min and rinsed with tap water. After staining, the sections were dehydrated by sequential immersion in 70% ethanol (2 min), 80% ethanol (2 min), 95% ethanol (2 min), 100% ethanol (twice, 2 min each), and xylene (twice, 5 min each). Finally, the dehydrated sections were mounted with neutral balsam and covered with coverslips. The stained sections were observed under a light microscope (Olympus Corporation).

Determination of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels. The serum levels of AST and ALT were measured in accordance with the guidelines provided by the AST Assay Kit (cat. no. C010-2-1) and ALT activity assay kit (cat. no. C009-2-1; both from Nanjing Jiancheng Bioengineering Institute). The absorbance was measured at 510 nm using a microplate reader (Thermo Fisher Scientific, Inc.).

Detection of malondialdehyde (MDA) levels. MDA levels in liver tissues were measured using MDA assay kit (cat. no. A003-1-2; Nanjing Jiancheng Bioengineering Institute) following the manufacturer's instructions.

Small interfering (siRNA) transfection. HepG2 and Huh-7 liver cancer cells were plated at a density of 3×10^5 cells/well in 6-well plates and cultured overnight. For siRNA transfection, 100 μ l of serum-free culture medium was first added to sterile centrifuge tubes. Subsequently, 12 μ l of a specific

siRNA targeting GLP-1R (si-GLP-1R, 5'-UAUUGGAAA ACAUUAUGCUU-3') or negative control (NC, 5'-UUC UCCGAACGUGUCACGUAA-3') was added to achieve a final concentration of 20 nM siRNA. In another tube, 12 μ l of HiPerFect Transfection Reagent (Qiagen China Co., Ltd.) was added to 100 μ l of serum-free culture medium. The contents of these two tubes were gently mixed and incubated at room temperature for 20 min to form the transfection complex. Next, the mixture was added to the wells containing the cells at 37°C for 6 h. The transfection mixture was then removed, and fresh DMEM containing 10% FBS was added, followed by incubation for 24 h.

Statistical analysis. The data are presented as the mean $\pm$ standard deviations (SDs). Two-tailed unpaired Student's t-tests were used to compare two groups, and one-way analysis of variance followed by Tukey's post hoc test was used to compare three or more groups. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

HP-B and fasting work together to counteract OA/PA-induced lipid accumulation in liver cells. CCK-8 analysis revealed that treatment with 10, 25 and 50 μ M HP-B did not affect the viability of HepG2 or Huh-7 liver cancer cells, whereas treatment with 75, 100 and 200 μ M HP-B reduced the viability of both cell types (Fig. 1A and B). Therefore, 50 μ M HP-B was used in subsequent experiments. Oil Red O staining revealed that OA/PA treatment markedly increased lipid accumulation in both cell types (Fig. 1C and D). However, HP-B treatment effectively attenuated OA/PA-induced lipid accumulation. Similarly, in HepG2 and Huh-7 liver cancer cells, fasting alone restored OA/PA-induced lipid accumulation, but HP-B treatment in addition to fasting significantly decreased lipid accumulation (Fig. 1C and D). After OA/PA treatment, quantitative analysis revealed that HepG2 and Huh-7 liver cancer cells had higher TC and TG levels than the control cells (Fig. 1E-H). Moreover, the increased TC and TG levels caused by OA/PA were reversed by HP-B treatment and fasting, and the combined treatment had a greater effect (Fig. 1E-H). Additionally, the mRNA levels of genes associated with lipid production and absorption (SREBP1, FAS and CD36) in HepG2 and Huh-7 liver cancer cells were quantitatively examined. OA/PA treatment significantly increased the mRNA levels of these genes compared with the control group (Fig. 1I and J). However, the OA/PA-induced increase in the mRNA levels of these genes was reduced by HP-B treatment and fasting, and the combination treatment had a more significant effect (Fig. 1I and J). In conclusion, OA/PA-induced lipid accumulation and related gene upregulation in HepG2 and Huh-7 liver cancer cells are successfully reversed by both HP-B treatment and fasting, with their combined use showing increased efficiency.

Combined HP-B and fasting treatment reverses the OA/PA-induced decrease in the MMP. Assessment of the ROS levels in HepG2 and Huh-7 liver cancer cells revealed that OA/PA treatment increased the ROS levels compared with those in the Con group. However, HP-B treatment and

fasting substantially reduced this increase in ROS levels in both the HepG2 and Huh-7 liver cancer cells (Fig. 2A-D). Notably, the combined treatment of HP-B and fasting further reduced the ROS levels (Fig. 2A-D). Compared with the controls, OA/PA treatment significantly increased the levels of mitoROS in HepG2 and Huh-7 liver cancer cells. Both HP-B treatment and fasting reduced the increase in mitoROS levels caused by OA/PA, and the combined treatment resulted in a more significant reduction (Fig. 2E-H). Additionally, OA/PA treatment significantly decreased the MMP in HepG2 and Huh-7 liver cancer cells. Both HP-B treatment and fasting reversed the OA/PA-induced reduction in the MMP, and the combined treatment further ameliorated this decrease (Fig. 2I and J).

Combined HP-B and fasting treatment reverses the OA/PA-induced decrease in GLP-1R and PGC1 α . To evaluate the effects of HP-B and fasting on the expression of GLP-1R and PGC1 α , the mRNA levels of GLP-1R and PGC1 α were examined in HepG2 and Huh-7 liver cancer cells. Compared with the Con group, OA/PA significantly decreased the mRNA levels of GLP-1R and PGC1 α , whereas HP-B treatment and fasting reversed the OA/PA-induced reduction in the mRNA levels of GLP-1R and PGC1 α (Fig. 3A-D). More importantly, the combination of HP-B treatment and fasting further increased the mRNA levels of GLP-1R and PGC1 α (Fig. 3A-D). Evaluation of the GLP-1R and PGC1 α protein expression levels in HepG2 and Huh-7 liver cancer cells revealed that OA/PA treatment significantly decreased the protein expression of GLP-1R and PGC1 α (Fig. 3E and F). However, HP-B treatment or fasting reversed the OA/PA-induced decrease in the protein expression of GLP-1R and PGC1 α , and the combined treatment of HP-B and fasting was more effective (Fig. 3E and F).

Combined HP-B and fasting treatment reverses lipid accumulation in the livers of HFD-fed mice. Compared with the Con group, the HFD group presented significantly impaired glucose tolerance and decreased insulin sensitivity, indicating HFD-induced IR (Fig. 4A and B). Both interventions improved glucose metabolism: Fasting monotherapy reduced GTT area under the curve (AUC) by 19.96 (vs. HFD: 96.84-76.88). HP-B monotherapy reduced GTT AUC by 32.69 (vs. HFD: 96.84-64.15). Similarly, ITT AUC decreased by 13.10 with fasting and 9.47 with HP-B alone (vs. HFD: 40.84) (Fig. 4B). Critically, combination therapy reduced GTT AUC by 61.91 (vs. HFD: 96.84-34.93) and decreased ITT AUC by 26.81 (vs. HFD: 40.84-14.03). This exceeded the sum of individual effects, specifically, GTT: $61.91 > (32.69 + 19.96) = 52.65$, and ITT: $26.81 > (9.47 + 13.10) = 22.57$, demonstrating synergistic reversal of IR (Fig. 4A and B). Furthermore, the combined HP-B and fasting treatment had a more pronounced effect, as evidenced by enhanced glucose clearance in the GTT and greater improvement in insulin sensitivity in the ITT, which suggested a synergistic effect of HP-B and fasting in mitigating IR and metabolic dysfunction (Fig. 4A and B). Compared with those of the control group, the livers of HFD-fed mice presented a substantial increase in lipid accumulation (Fig. 4C and D). HP-B treatment or fasting

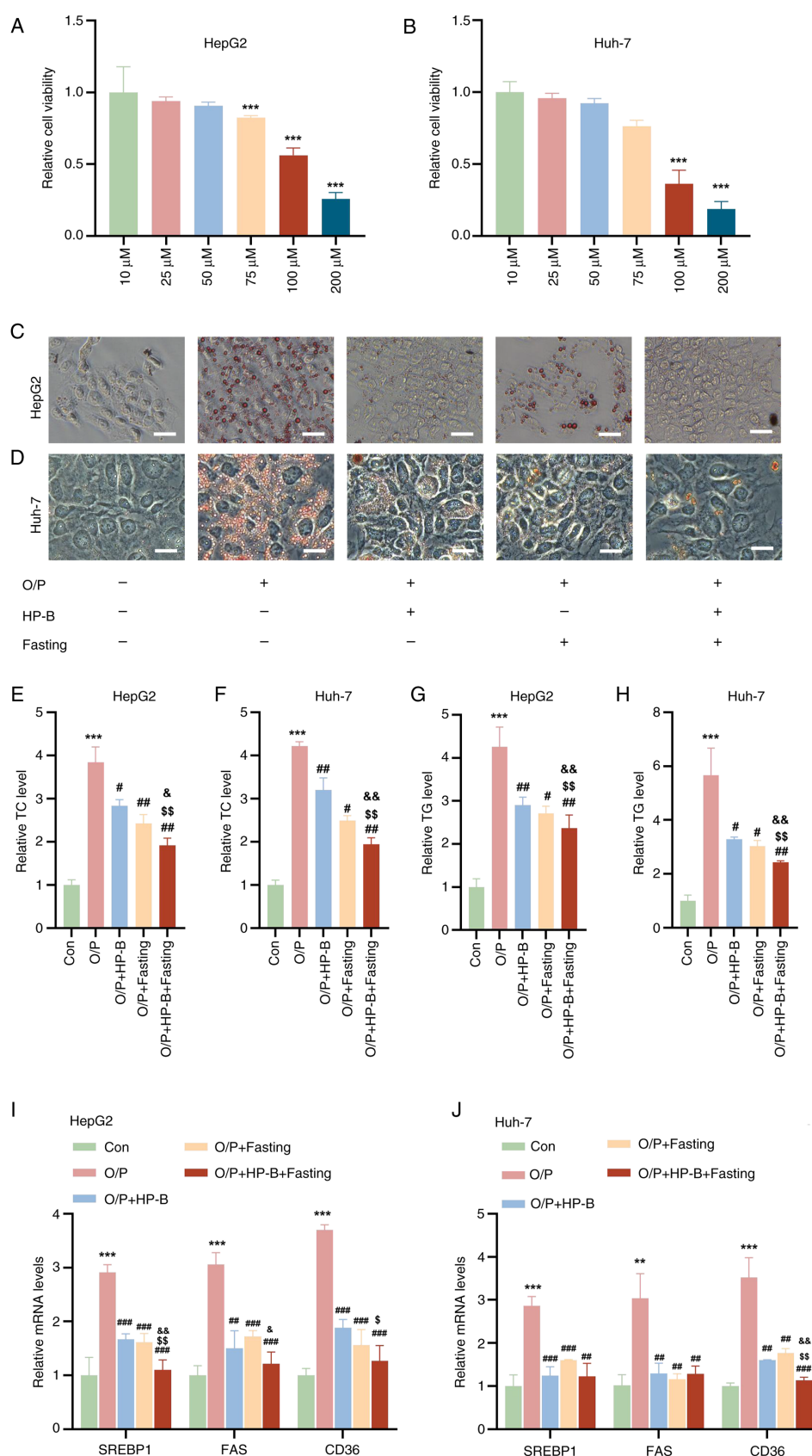


Figure 1. Effects of HP-B treatment and fasting on OA/PA-induced lipid accumulation and gene expression in HepG2 and Huh-7 liver cancer cells. (A and B) Cell Counting Kit-8 analysis revealed that 10, 25 and 50 μ M HP-B did not significantly alter cell viability, whereas 75, 100 and 200 μ M HP-B reduced cell viability in HepG2 and Huh-7 cells. (C and D) Oil Red O staining of HepG2 and Huh-7 liver cancer cells treated with OA/PA revealed significant lipid accumulation compared with the Con group. Both HP-B treatment and fasting reversed OA/PA-induced lipid accumulation, and the combined HP-B and fasting treatment further reduced lipid accumulation (Scale bar, 10 μ m). (E-H) Quantitative analysis of TC and TG levels in HepG2 and Huh-7 liver cancer cells. (I and J) Quantitative analysis of the mRNA levels of lipid synthesis- and uptake-related genes (SREBP1, FAS and CD36) in HepG2 and Huh-7 liver cancer cells. ** P <0.01 and *** P <0.001 vs. Con; # P <0.05, ## P <0.01 and ### P <0.001 vs. OA/PA; \$ P <0.05 and \$\$ P <0.01 vs. OA/PA + Fasting. HP-B, heterophyllin B; OA/PA, oleic acid/palmitic acid; TC, total cholesterol; TG, triglycerides.

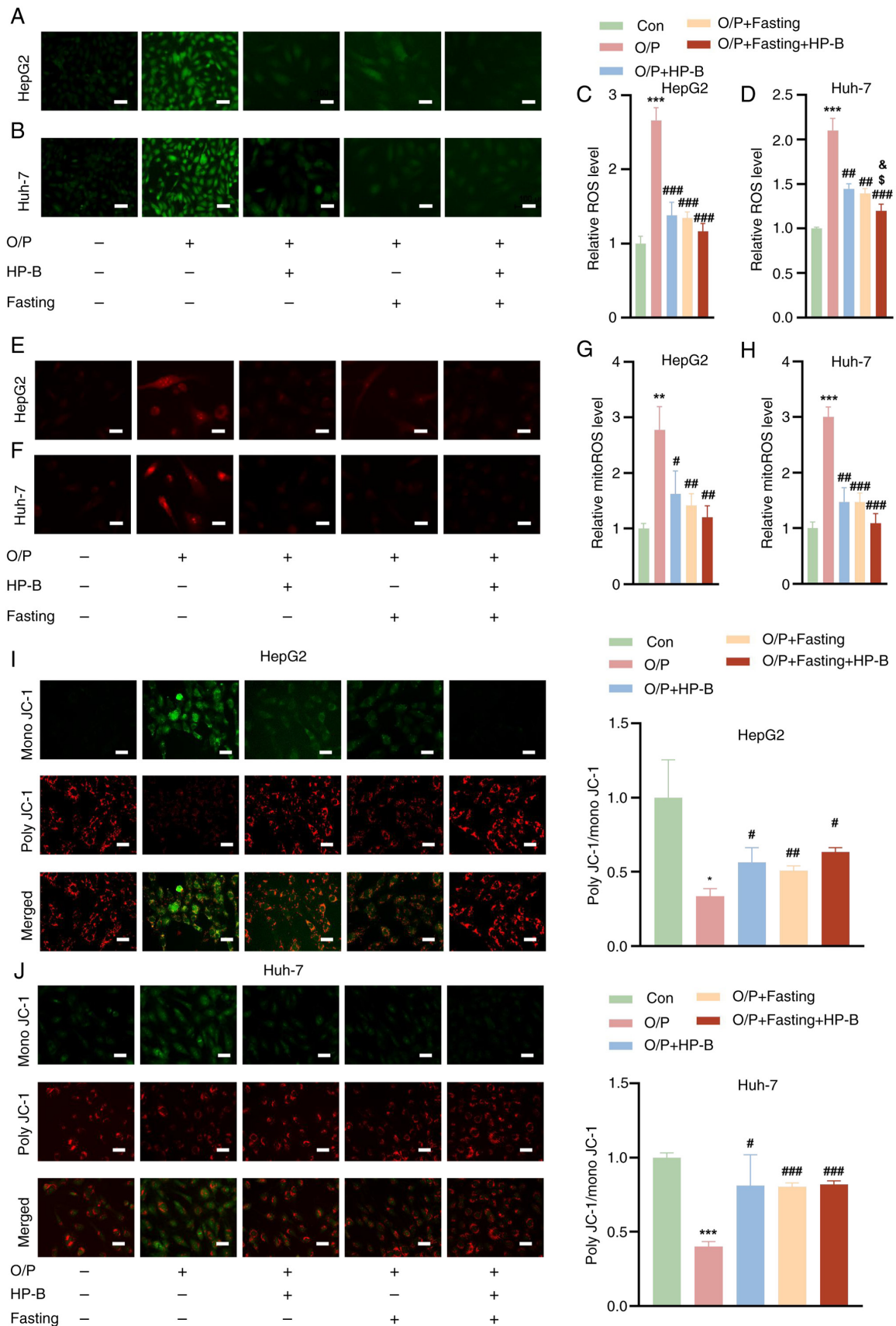


Figure 2. Effects of HP-B treatment and fasting on OA/PA-induced changes in ROS, mitoROS, and the MMP in HepG2 and Huh-7 liver cancer cells. (A and B) Representative images of the ROS levels in (A) HepG2 and (B) Huh-7 cells are shown. (C and D) Quantitative analysis of ROS levels in (C) HepG2 and (D) Huh-7 cells. (E and F) Representative images and quantification of mitoROS levels in (E) HepG2 and (F) Huh-7 cells are shown. (G and H) Quantitative analysis of mitoROS levels in (G) HepG2 and (H) Huh-7 cells. (I and J) Representative images and quantification of the MMP in (I) HepG2 and (J) Huh-7 cells are shown. Scale bar, 10 μ m. * P <0.05, ** P <0.01 and *** P <0.001 vs. Con; # P <0.05, ## P <0.01 and ### P <0.001 vs. OA/PA; \$ P <0.05 vs. OA/PA + Fasting. HP-B, heterophyllin B; OA/PA, oleic acid/palmitic acid; ROS, reactive oxygen species; mitoROS, mitochondrial ROS; MMP, mitochondrial membrane potential.

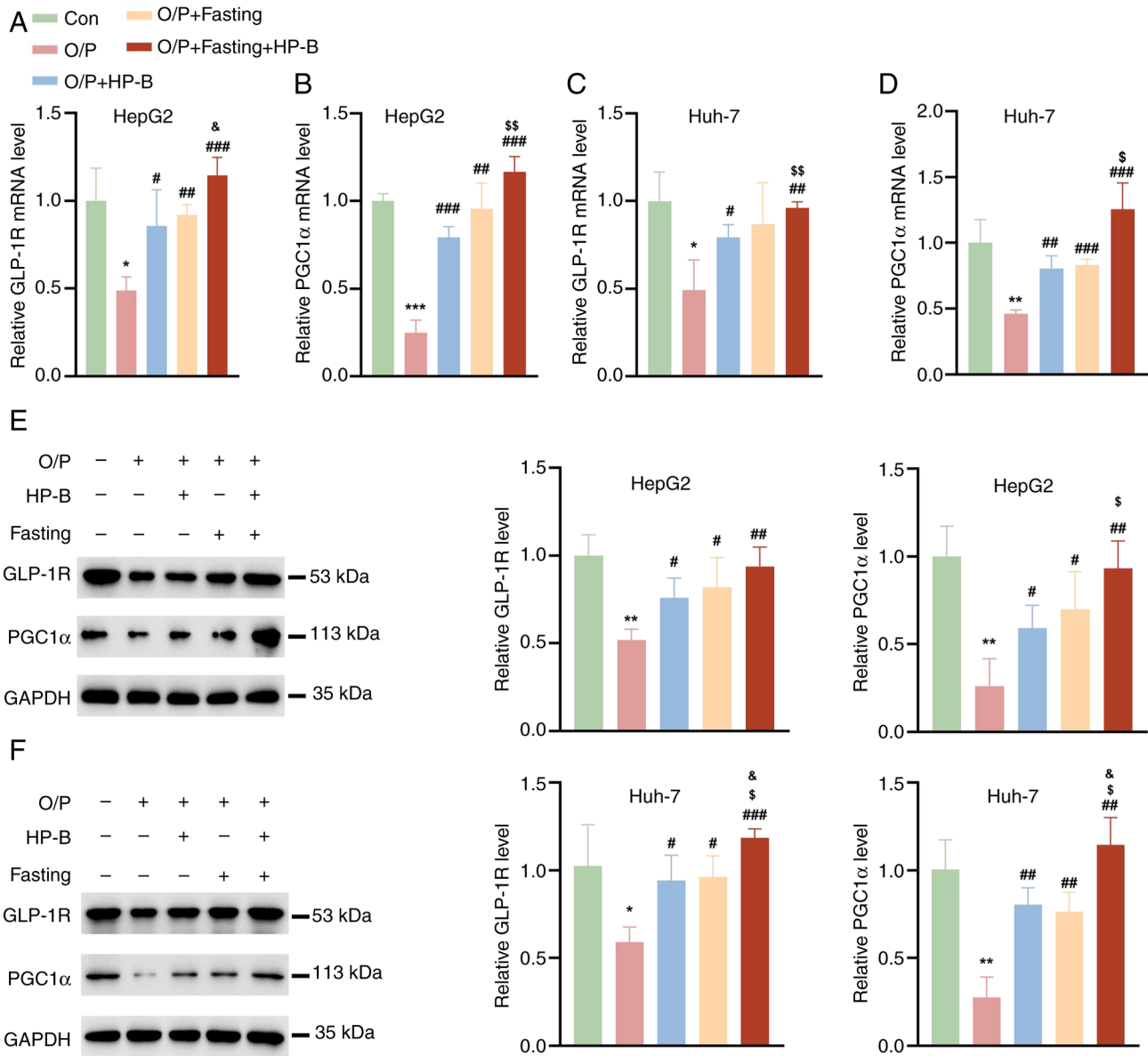


Figure 3. Effects of HP-B treatment and fasting on OA/PA-induced changes in GLP-1R and PGC1 α expression in HepG2 and Huh-7 liver cancer cells. (A-D) Quantitative analysis of GLP-1R and PGC1 α mRNA levels in HepG2 and Huh-7 liver cancer cells. (E and F) HP-B treatment or fasting reversed the OA/PA-induced reduction in protein levels, and the combined treatment had a more pronounced effect. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs. Con; # $P < 0.05$, ## $P < 0.01$ and ### $P < 0.001$ vs. OA/PA; \$ $P < 0.05$ and \$\$ $P < 0.01$ vs. OA/PA + HP-B; & $P < 0.05$ vs. OA/PA + Fasting. HP-B, heterophyllin B; OA/PA, oleic acid/palmitic acid; GLP-1R, glucagon-like peptide-1 receptor; PGC1 α , peroxisome proliferator-activated receptor gamma coactivator 1- α .

reduced hepatic lipid accumulation, and the combined HP-B and fasting further reduced lipid levels (Fig. 4C and D). Compared with those in the control group, the mice in the HFD group had significantly greater liver weights, body weights and liver indices (Fig. 4E-G). Fasting effectively reduced the liver weight, body weight and liver index in HFD-fed mice, whereas HP-B treatment specifically reduced the liver weight and liver index (Fig. 4E-G). Hepatic steatosis was profoundly induced by HFD, with TG content surging to 64.74 ± 7.23 mg/g wet weight vs. 5.127 ± 0.62 mg/g in controls (Fig. 4H). Monotherapies partially attenuated this accumulation: HP-B alone reduced TG by 21.65 mg/g (HFD + HP-B: 43.09 ± 1.54 mg/g), while fasting alone reduced TG by 15.30 mg/g (HFD + Fasting: 49.44 ± 6.113 mg/g). Critically, the combination therapy achieved a TG reduction

of 45.79 mg/g (HFD + Fasting + HP-B: 18.95 ± 3.70 mg/g), significantly exceeding the additive effect of monotherapies ($21.65 + 15.30 = 36.95$ mg/g) (Fig. 4H). This enhancement beyond additivity demonstrates synergistic amelioration of hepatic lipidosis. Additionally, the HFD group presented significantly elevated levels of AST and ALT compared with those in the control group. The HP-B and fasting treatments clearly reduced these indices (Fig. 4I and J). The protein expression levels of GLP-1R and PGC1 α in the livers of HFD-fed mice were significantly lower than those in the control group (Fig. 4K). HP-B treatment or fasting increased the expression of these proteins, and the combined treatment exerted a more significant effect on PGC1 α protein expression (Fig. 4K). These findings suggested that the combination of HP-B treatment and fasting may exert a

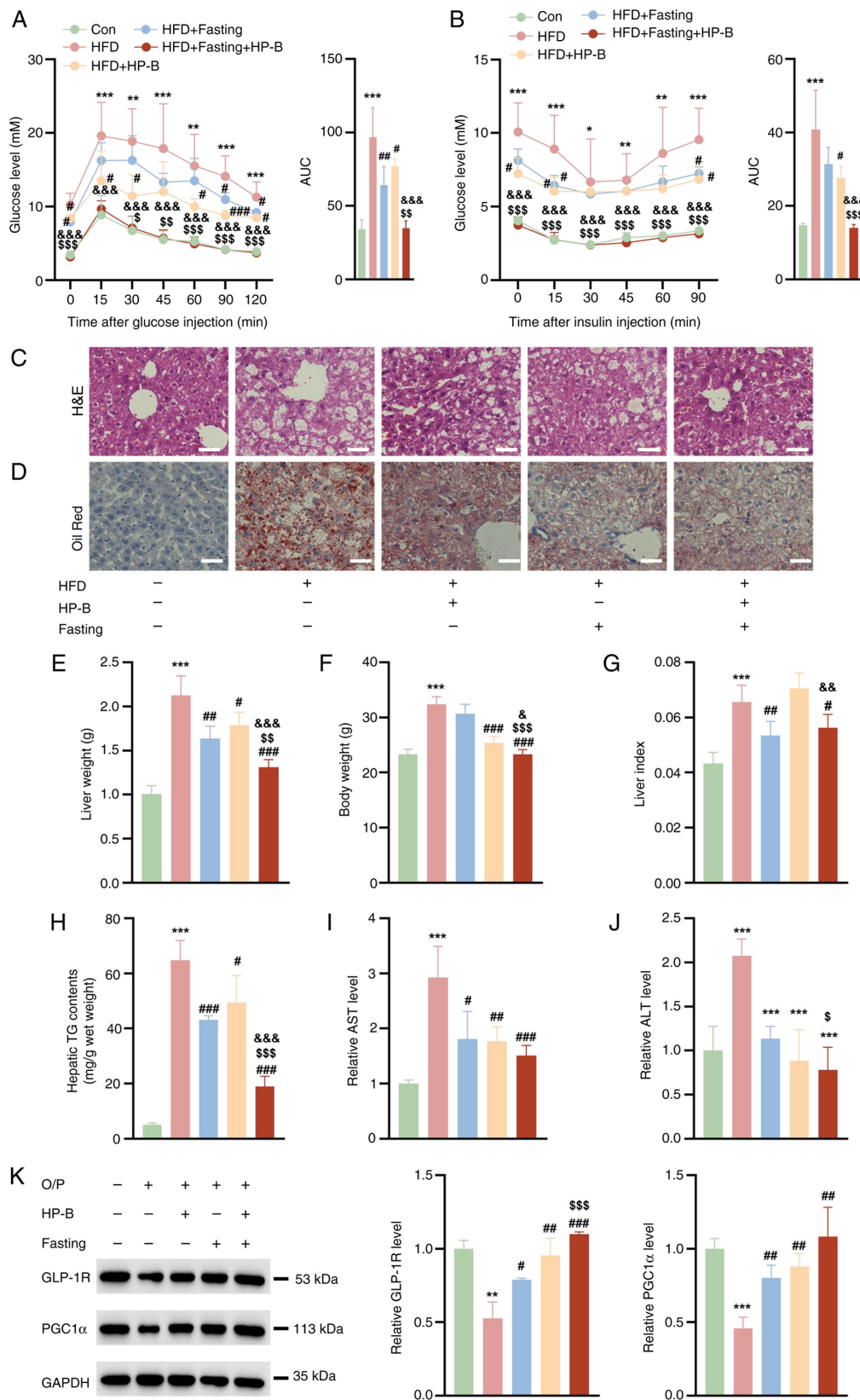


Figure 4. Effects of HP-B treatment and fasting on lipid accumulation and related parameters in the livers of HFD-fed mice. (A) Glucose tolerance test and AUC quantification. (B) Insulin tolerance test and AUC quantification. (C and D) Representative H&E and Oil Red O images showing that HP-B treatment or fasting treatment reduced liver lipid accumulation to some extent, whereas the combined HP-B and fasting treatment further significantly reduced lipid accumulation (Scale bar, 25 μ m). (E-G) HP-B treatment or fasting reduced the liver weight, body weight and liver index. The combined treatment resulted in a more pronounced reduction in liver weight and body weight, but the liver index did not obviously change. (H-J) HP-B treatment and fasting lowered these levels, and the combined treatment resulted in a more significant reduction in TG and ALT levels than HP-B treatment alone, as well as a more significant reduction in TG levels than fasting alone. (K) HP-B treatment or fasting increased the protein levels of GLP-1R and PGC1 α , and the combined treatment further increased PGC1 α protein expression compared with HP-B treatment alone. * P <0.05, ** P <0.01 and *** P <0.001 vs. Con; # P <0.05, ## P <0.01 and ### P <0.001 vs. HFD; \$ P <0.05, \$\$ P <0.01 and \$\$\$ P <0.001 vs. HFD + HP-B; & P <0.05, && P <0.01 and &&& P <0.001 vs. HFD + Fasting. HP-B, heterophyllin B; HFD, high-fat diet; AUC, area under the curve; TG, triglycerides; ALT, alanine aminotransferase; AST aspartate aminotransferase; GLP-1R, glucagon-like peptide-1 receptor; PGC1 α , peroxisome proliferator-activated receptor gamma coactivator 1-alpha.

synergistic effect on hepatic lipid metabolism and overall liver function.

GLP-1R silencing reverses OA/PA-induced lipid accumulation in HepG2 and Huh-7 liver cancer cells. si-GLP-1R or NC were then transfected into HepG2 and Huh-7 cells which were not combined with any other treatments. RT-qPCR analysis showed that the mRNA level of GLP-1R was decreased in HepG2 and Huh-7 cells compared with those of NC (Fig. 5A and C). To examine whether the combination of HP-B treatment and fasting ameliorates OA/PA-induced lipid accumulation in hepatocytes via GLP-1R, siRNAs specifically targeting SLP-1R were used. Compared with the Con group, si-GLP-1R significantly reduced GLP-1R expression in HepG2 and Huh-7 liver cancer cells (Fig. 5B and D). Moreover, GLP-1R silencing reduced downstream PGC1 α protein expression (Fig. 5B and D). GLP-1R silencing significantly decreased the protein level of GLP-1R and suppressed PGC1 α expression in HepG2 and Huh-7 liver cancer cells pretreated with OA/PA (Fig. 5B and D). Oil red O staining revealed that while OA/PA-induced lipid accumulation was mitigated by fasting and HP-B treatments, this ameliorative effect was prevented by PGC1 α silencing (Fig. 5E). Furthermore, in HepG2 and Huh-7 liver cancer cells, HP-B treatment and fasting decreased the TG and TC levels, but GLP-1R silencing further increased the TG and TC levels in these cells (Fig. 5F-I). Hence, GLP-1R silencing restores OA/PA-induced lipid accumulation, suggesting a possible mechanism by which hepatocyte lipid metabolism is improved by a combination of HP-B treatment and fasting via GLP-1R.

GLP-1R knockdown reverses the hepatoprotective effects of fasting and HP-B treatment in HFD-fed mice. As shown in Fig. 6A, GLP-1R was effectively silenced in the livers of HFD + Ad-NC and HFD + Fasting + HP-B + Ad-NC mice. GLP-1R silencing abolished the beneficial effects of HP-B treatment and fasting on glucose metabolism, as evidenced by impaired GTT and reduced ITT, with curves approaching those of the HFD group (Fig. 6B and C). Knocking down GLP-1R abolished the protective benefits of HP-B and fasting in HFD-fed mice. Compared with HFD + Ad-NC mice alone, Ad-short hairpin (sh)GLP-1R exacerbated lipid accumulation in the livers of HFD-fed mice (Fig. 6D and E). Silencing GLP-1R prevented the combined effect of HP-B treatment and fasting from reducing lipid droplet deposition, even in HFD-fed animals subjected to these interventions (Fig. 6D and E). While GLP-1R knockdown did not significantly affect the body weight of HFD-fed mice, it increased their liver weight (Fig. 6F and G). Similarly, compared with those in the fasting and HP-B treatment groups, the liver weights of the GLP-1R-deficient mice were greater, but their body weights did not appreciably change (Fig. 6F and G). Compared with the HFD + Ad-NC group, the hepatic indices of HFD-fed mice after fasting and HP-B treatment were lower, but these effects were reversed when GLP-1R was silenced (Fig. 6H). Compared with that in the HFD + Ad-NC group, the mouse liver TG content was lower after GLP-1R knockdown, but this effect was minimized with fasting and HP-B treatment after silencing GLP-1R (Fig. 6I). Additionally, GLP-1R silencing reversed the reductions in

ALT and AST levels induced by fasting and HP-B treatment (Fig. 6J and K). Compared with those in the HFD + Ad-NC group, the MDA and ROS levels in the livers of HFD-fed mice were lower after fasting and HP-B treatment (Fig. 6L and M). Conversely, Ad-shGLP-1R injections into the tail vein of HFD-fed mice resulted in elevated levels of MDA and ROS in their livers (Fig. 6L and M). These findings indicated that GLP-1R is essential for the metabolic improvements mediated by HP-B treatment and fasting.

Discussion

Previous studies have demonstrated that IF effectively improves MASLD by modulating metabolism, reducing fat accumulation, and mitigating inflammatory responses (5,21). However, prolonged fasting may lead to potential side effects, such as hypoglycemia and nutritional deficiencies, and the long-term efficacy remains to be further validated (22). Therefore, the inclusion of natural compounds with hepatoprotective properties, such as HP-B, may enhance the therapeutic effects of IF while mitigating its adverse outcomes. HP-B has significant anti-inflammatory, antioxidant and immunomodulatory activities, and antitumor effects, making it a promising candidate for improving liver function and providing additional benefits in the management of MASLD (14-16). Further investigation into the synergistic effects of HP-B treatment combined with IF is warranted to optimize treatment strategies for MASLD.

In the context of metabolic disease therapeutics, GLP-1R agonists have emerged as key pharmacologic agents because of their multifaceted benefits in glycemic control, weight reduction and cardiovascular risk mitigation (23,24). These strategies offer distinct advantages as follows: i) multitarget synergy enables greater weight loss and comprehensive metabolic restoration, and ii) potential to delay or reverse multiorgan pathologies such as MASLD. Nevertheless, co-agonists present several challenges. Tolerability remains a concern, as effective doses in animal models frequently induce gastrointestinal adverse events (for example, nausea and vomiting) in humans, which are particularly problematic for GLP-1-based therapies. Additionally, the energy expenditure mechanisms mediated via sympathetic nervous system activation and brown adipose tissue thermogenesis are robust in rodents but often fail to translate clinically (23,24). Further complexities include intricate mechanism-of-action profiles, difficulties in optimizing receptor activation ratios, and undefined cardiovascular safety risks.

Previous research on HP-B has focused primarily on its anti-inflammatory, antioxidant and immunomodulatory activities, with applications largely confined to tumor suppression, neurodegenerative diseases and cognitive disorders (16,20,25). However, the potential therapeutic value of HP-B in MASLD has received limited attention. The present study provides several novel contributions. First, the present study provides the first experimental evidence supporting the synergistic efficacy of HP-B treatment combined with IF in alleviating MASLD-related metabolic disturbances. Second, the present findings revealed a previously uncharacterized mechanism by which HP-B exerts its metabolic benefits. Specifically, GLP-1R-knockdown models confirmed the central role of the GLP-1R/PGC1 α signaling axis in mediating the protective

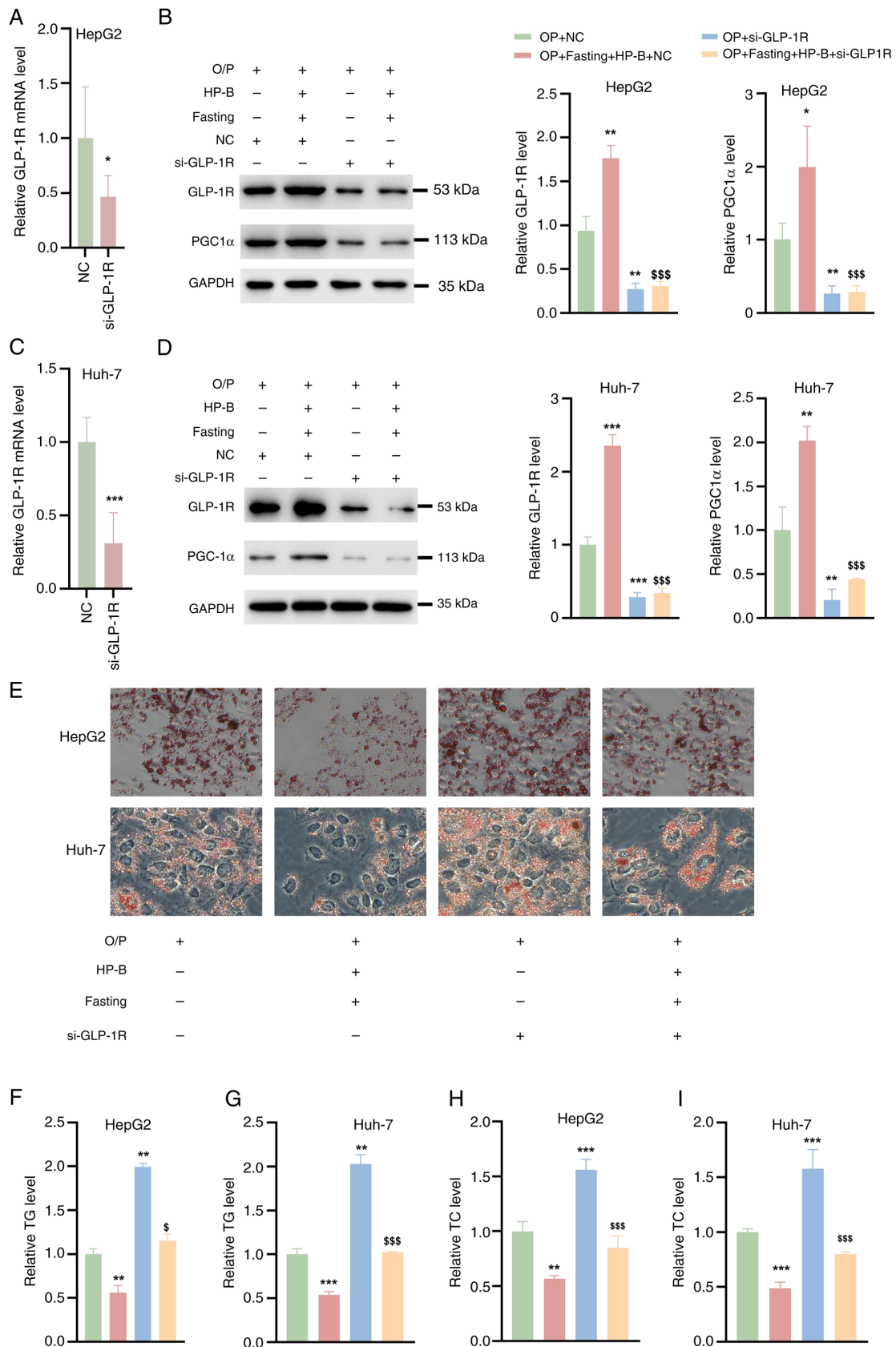


Figure 5. Effects of GLP-1R silencing on OA/PA-induced lipid accumulation in HepG2 and Huh-7 liver cancer cells. (A and C) Reverse transcription-quantitative PCR analysis showed that the mRNA levels of GLP-1R in HepG2 and Huh-7 cells were reduced after siGLP-1R transfection. (B and D) Western blot analysis revealed that siGLP-1R silenced GLP-1R expression in OA/PA pre-treated HepG2 and Huh-7 cells and reversed the OA/PA-induced increase in PGC1α protein levels. (E) Oil Red O staining demonstrated that fasting and HP-B treatment reduced lipid accumulation induced by OA/PA, but this effect was blocked by GLP-1R silencing. (F-I) GLP-1R silencing further elevated (F and G) TG and (H and I) TC levels in HepG2 and Huh-7 cells, counteracting the reductions achieved by fasting and HP-B treatment. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs. OA/PA; $^{\$}P < 0.05$ and $^{$$$}P < 0.01$ vs. OA/PA + HP-B + Fasting. HP-B, heterophyllin B; GLP-1R, glucagon-like peptide-1 receptor; OA/PA, oleic acid/palmitic acid; si-, small interfering; TG, triglycerides; TC, total cholesterol; NC, negative control.

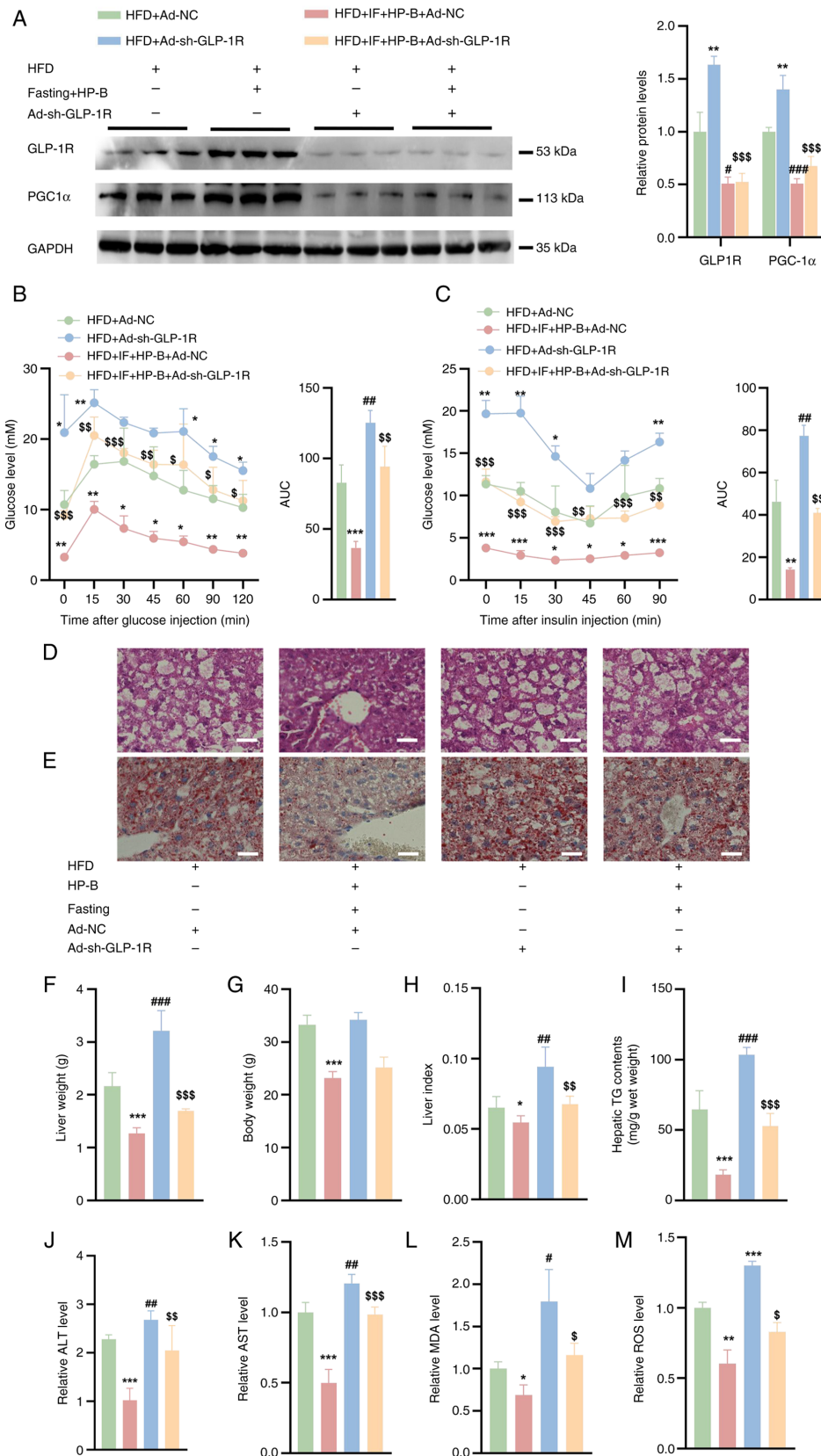


Figure 6. GLP-1R knockdown impacts fasting and HP-B treatment in HFD + Ad-NC mice. (A) Western blot analysis showed that the expression of GLP-1R was decreased in the livers of HFD mice. (B and C) Glucose tolerance test and insulin tolerance test. (D and E) H&E staining and Oil Red O staining of liver sections from HFD-fed mice (Scale bar, 25 μ m). (F and G) Liver weights and body weights of HFD-fed mice. (H) Compared with the oil/control treatment, fasting and HP-B treatment reduced the liver index, whereas GLP-1R knockdown reversed this effect. (I) GLP-1R knockdown reduced the TG content compared with that in HFD-fed mice, and this effect was counteracted by fasting and HP-B treatment. (J and K) Fasting and HP-B treatment decreased the ALT and AST levels, and these reductions were reversed by GLP-1R knockdown. (L and M) Fasting and HP-B treatment reduced the MDA and ROS levels, whereas Ad-shGLP-1R injection increased these levels. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs. HFD + Ad-NC; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ vs. HFD + Ad-sh-GLP-1R; \$ $P < 0.05$, \$\$ $P < 0.01$ and \$\$\$ $P < 0.001$ vs. HFD + HP-B + IF. GLP-1R, glucagon-like peptide-1 receptor; HP-B, heterophyllin B; HFD, high-fat diet; NC, negative control; TG, triglycerides; ALT, alanine aminotransferase; AST aspartate aminotransferase; MDA, malondialdehyde; ROS, reactive oxygen species; sh-, short hairpin.

effects of HP-B. These findings significantly broaden the pharmacological profile of HP-B and suggest a new conceptual and therapeutic framework for MASLD intervention.

Previous studies have shown that the activation of GLP-1R regulates lipid metabolism and mitochondrial homeostasis via the AMPK/PGC1 α signaling axis (26,27). For example, in a spinal cord injury model, the exendin9-39 GLP-1R antagonist and the SR18292 PGC1 α inhibitor have been used to functionally validate the GLP-1R/AMPK/PGC1 α signaling pathway (26). Similarly, in obesity-related chronic kidney disease models, the liraglutide GLP-1R agonist reduces renal lipid deposition and restores mitochondrial function through the Sirt1/AMPK/PGC1 α axis (27). These studies collectively suggest that AMPK may act as a critical intermediary linking GLP-1R activation to PGC1 α -mediated metabolic protection. To further validate this hypothesis in the context of HP-B and IF, the authors plan to employ pharmacologic inhibitors of AMPK (for example, Compound C) and generate conditional AMPK knockout models. These strategies will help elucidate the downstream signaling network of GLP-1R and clarify the mechanistic basis of HP-B-mediated metabolic regulation.

To ensure the reproducibility and physiological relevance of the present results, a well-structured and widely accepted 5:2 IF protocol was adopted. Prior studies have shown that this regimen activates glucocorticoid (GC) signaling, which contributes to metabolic reprogramming by upregulating Pck1 expression and enhancing fatty acid oxidation and gluconeogenesis (4). Importantly, this GC activation is considered an adaptive physiological response rather than a marker of stress pathology. In support of this notion, 5:2 IF has been reported to have minimal effects on anxiety-like behaviors or adult hippocampal neurogenesis in mice (28), suggesting that it is behaviorally and neurologically well tolerated. Although the present study did not include direct measurements of serum corticosterone levels at fasting endpoints due to logistical limitations, the authors plan to incorporate such assessments in future experiments to systematically evaluate stress-related responses and control for their potential confounding impact.

Despite these promising findings, the present study has certain limitations. Although reproducible, the OA/PA-induced *in vitro* model of hepatic lipid accumulation may not fully capture the complexity of MASLD pathogenesis. In future studies, it is intended to incorporate additional proinflammatory stimuli, such as TNF- α or lipopolysaccharide, to better mimic the inflammatory milieu of the disease and increase the robustness of the findings. Although GLP-1R knockdown significantly attenuated the beneficial effects of HP-B treatment and IF, it did not completely abolish them, which suggests the existence of GLP-1R-independent pathways, potentially involving direct AMPK activation or the modulation of oxidative stress responses. Thus, further investigation is warranted. In addition, the present study did not assess the pharmacokinetic properties of HP-B, such as its bioavailability, half-life and tissue distribution. These parameters are crucial for understanding the *in vivo* efficacy and safety profile of HP-B. Without specific data, it is challenging to predict its behavior in biological systems accurately. Future studies should aim to characterize the pharmacokinetic parameters

of HP-B to better understand its therapeutic potential and optimize dosing regimens. Moreover, while our data demonstrate functional interaction between HP-B and the GLP-1R pathway, it is acknowledged that direct molecular binding remains uncharacterized. To address this mechanistic gap, molecular docking or receptor binding assays will be carried out as future directions.

In summary, the present study elucidates the mechanism by which HP-B treatment improves lipid metabolism, protects mitochondrial function, and alleviates oxidative stress through activation of the GLP-1R/PGC1 α axis. The combination of HP-B treatment and IF has significant synergistic effects and offers a low-toxicity, mechanistically grounded intervention strategy for the treatment of MASLD.

Acknowledgements

Not applicable.

Funding

The present study was supported by the Beijing University of Chemical Technology-China-Japan Friendship Hospital Biomedical Transformation Engineering Research Center Joint Fund Project (grant no. XK2022-07).

Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

KL performed the experiments and wrote the manuscript. LD and LX performed the experiments and interpreted the data. SY supervised the project, interpreted the data, and wrote the manuscript. KL and SY confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The experimental protocol was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Beijing University of Aeronautics and Astronautics (approval no. 2023-KY-064-1; Beijing, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Use of artificial intelligence tools

During the preparation of this work, artificial intelligence tools were used to improve the readability and language of the manuscript or to generate images, and subsequently, the

authors revised and edited the content produced by the artificial intelligence tools as necessary, taking full responsibility for the ultimate content of the present manuscript.

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