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Egg white protein alleviated high-fat-diet-induced obesity in mice by modulating lipid metabolism and gut microbiota: novel insights into synergistic effect

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ABSTRACT: Egg white protein (EWP) is considered an optimal dietary protein for weight management due to its superior amino acid profile, high biological value, and diverse metabolic regulatory functions. The study explored the effects of EWP on lipid metabolism in high-fat diet (HFD)-induced obese mice through transcriptomic and gut microbiota analyses. These results showed that EWP intake significantly influenced body weight, adipose tissue, and glucose tolerance in obese mice. EWP-1200 reduced blood lipid characteristics, decreased adipocyte size and improved hepatic injury. Moreover, EWP supplement elevated total fecal lipid, triglyceride, and total cholesterol levels in HFD-induced obese mice, concurrently suppressing fecal lipase activity. These findings suggested that EWP might enhance lipid excretion and inhibit lipid absorption. Liver transcriptomics and RT-qPCR analyses revealed that EWP significantly enhanced mRNA expression within the hepatic peroxisome proliferator-activated receptor (PPAR) signaling pathway. Notably, EWP exerted significant regulatory effects on genes such as CD-36, HSL, PPAR α , FASN, and ACC, indicating that EWP might mitigate obesity by modulating lipid metabolism pathways mediated through PPAR signaling. Additionally, gut microbiota studies indicated that EWP decreased the *Firmicutes/Bacteroidetes* ratio, enhanced the abundance of *Akkermansia*, *Lactobacillus*, and *Bifidobacterium*, and significantly improved gut microbiota diversity and richness, thereby substantiating EWP's weight-loss effects on HFD-obese mice. The study clarified the efficacy and potential of egg white protein as a dietary intervention for obesity in mice, offering a scientific basis for its high value.

Keywords: Egg white protein; High-fat diet; Obesity; Transcriptome; Gut microbiota; Lipid regulation

1. Introduction

Obesity, as a noninfectious chronic metabolic disease characterized by excessive body fat, has received widespread global attention. According to the World Health Organization, the global prevalence of obesity more than doubled from 1990 to 2022, and with obesity rates continuing to rise, relevant projections indicated that over 1.5 billion adults would be classified as obese by 2035. Obesity not only disrupted lipid metabolism but also triggered a cascade of complications, including hypertension, type 2 diabetes, inflammation, and

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osteoporosis^[3, 4]. The primary causes of obesity included a sedentary lifestyle and excessive calorie intake, underscoring the growing demand for safe and effective treatment strategies^[5, 6]. Pharmaceutical interventions and lifestyle changes have been effective in alleviating obesity, but their long-term effectiveness is often restricted, and concerns about associated side effects still maintain. Pharmacological interventions, a prevalent method for addressing obesity, have been successful in reducing ectopic fat buildup, showing promise for lipid control. However, the attention has been commonly reported to notable adverse effects including constipation, indigestion, and drowsiness. Consequently, the identification of natural bioactive compounds with anti-obesity properties has been of considerable importance.

Protein, a vital dietary bioactive substance, has garnered significant attention for its role in obesity prevention and is acknowledged as a safe and effective strategy^[8-12]. Liu et al.^[13] demonstrated that pea protein supplementation reduced weight gain and fat accumulation in mice induced by a high-fat diet (HFD), reversed lipid levels, and alleviated fatty liver degeneration. Cooked soybean protein peptides in the diet may inhibit pancreatic lipase activity, thereby delaying fat digestion and absorption to potentially alleviate obesity. Mung bean protein also served as a dietary protein source for obesity mitigation, effectively lowering blood lipid levels and reducing liver damage in mice. Consumption of fish skin collagen inhibited fat accumulation in HFD mice and downregulated the expression of fatty acid synthesis proteins in the liver. Whey protein, a highly nutritious protein, exhibited not only physiological functions such as antibacterial, anti-inflammatory, and antiviral effects after digestion and absorption, but also demonstrated potential for alleviating obesity^[17, 18], thereby playing a crucial role in human health. Egg white protein (EWP), a key high-quality protein, was characterized by its balanced amino acid profile and high nutritional value. Consequently, EWP was reported to possess physiological activities including antioxidant, anti-inflammatory, antibacterial, and antihypertensive effects. Additionally, its potential to regulate lipid metabolism aroused increasing attention^[21, 22]. Researches suggested that EWP could counteract weight gain, decrease adipose tissue and liver weight in HFD-induced obese mice. Meanwhile, EWP showed the capacity to reduce intestinal inflammation, enhance gut microbiota diversity^[24, 25], and improve glucose metabolism in obese mice. Consequently, EWP might alleviate hepatic lipid accumulation in HFD-obese mice by modulating lipid metabolism, enhancing gut microbiota, and improving glucose metabolism. Based on these findings, it was proposed that augmenting EWP consumption could represent a viable strategy against obesity. Nonetheless, the effectiveness and mechanisms of EWP intervention in HFD-induced obese mice require further investigation.

In summary, despite the increasing recognition of the potential role of dietary protein in addressing obesity, systematic investigations on the efficacy and mechanisms of EWP in alleviating obesity still have been limited. The study utilized the HFD-induced C57BL/6J obese mice model to explore the effects of EWP intervention on lipid metabolism. Furthermore, to elucidate the mechanisms underlying the anti-obesity effects of EWP, we performed comprehensive analyses of hepatic transcriptomes and gut microbiota. RT-qPCR was then used to validate the expression of key genes involved in lipid metabolism, providing insight into the molecular pathways through which EWP modulates lipid metabolism in HFD-induced obese mice. The

research could offer a theoretical support for utilizing egg white protein as a functional food for reducing obesity and holding significant importance in developing innovative anti-obesity dietary interventions.

2. Materials and Methods

2.1 Materials and Reagents

Fresh eggs were provided by Anhui Rongda Food Co., Ltd. (Guangde, China); Cholesterol (TC), triglyceride (TG), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), alanine aminotransferase (ALT), and aspartate aminotransferase (AST), superoxide dismutase (SOD), catalase (CAT), and malondialdehyde (MDA) assay kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China); 4% Polytetrafluoroethylene was purchased from Biosharp, Co., Ltd. (Beijing, China). Inflammatory factor IL-6, IL-1 β , and TNF- α ELISA development kits were purchased from Solarbio Science & Technology Co., Ltd. (Beijing, China). Sinocare GA-3 blood glucose test strips were purchased from Sinocare Biosensor Co., Ltd. (Changsha, China). Antibodies used in immunohistochemistry were purchased from ABclonal Technology (Wuhan, China), qPCR reagents were acquired from Yeasen Biotechnology Co., Ltd. (Shanghai, China). All other chemicals were analytical-grade reagents.

2.2 Preparation of Egg White Protein

Fresh eggs were wiped clean, and egg whites were carefully separated from fresh eggs. The egg whites were subsequently freeze-dried and stored at -20°C for subsequent experimental use.

2.3 Animal Experiment Design

The animal experiment was carried out with the approval from the Animal Protection Committee of the School of Food and Biological Engineering at Hefei University of Technology (No. HFUT20240405004), and all procedures were strictly performed in accordance with the ARRIVE guidelines to ensure the normativity and reproducibility of the experiment. A total of 108 male C57BL/6J mice with the age of 4 weeks were utilized in this study. The mice were obtained from Jiangsu Jicui Yaokang Biotechnology Co., Ltd. (Nanjing, China). They were maintained in a sterile environment with an ambient temperature of (22 $\pm$ 2)°C, humidity of 50% $\pm$ 5%, and a 12-hour light cycle (08:00-20:00), Food and water were provided ad libitum throughout the entire experimental period.

After a 1-week acclimatization period, mice with a consistent body weight of (18 $\pm$ 3.0) g were selected to minimize the interference of initial body weight on the subsequent obesity modeling, and the qualified mice were randomly allocated into nine groups using a random number table method. One group received a normal diet (referred to as the control group), while the remaining eight groups were fed a high-fat diet. Refer to Supplementary Table S1 for details on the feed compositions. After six weeks, the HFD groups exhibited a 20% increase in body weight, thereby confirming the successful establishment of the obesity model. Starting from the 7th week, the HFD group was subjected to varying protein intervention doses (as illustrated in Figure 1A). The interventions included the EWP group with five dose gradients: 300, 600, 900, 1200, and 1500 mg/(kg·d), denoted as EWP-300 to EWP-1500 respectively, the WP group at 600 mg/(kg·d), and the orlistat

group at 30 mg/(kg·d)-orlistat was selected as the positive control due to its well-established role as a clinically approved anti-obesity drug that inhibits intestinal fat absorption, wide application in HFD-induced obesity animal models for result comparison, and ability to validate the effectiveness of the experimental model. Conversely, the model group, while on an HFD, did not undergo any protein intervention. Moreover, both the model group and the control group were administered an equal volume of saline. The mice were monitored totally for 12 weeks with weekly assessments of body weight and food consumption. After the experimental period, the mice underwent a 12-hour fast before blood sampling from the orbital sinus. Blood samples were left at 4°C for 12 h, followed by centrifugation at 3000 g for 10 min. The resulting serum was stored at -80°C. Subsequently, the liver, spleen, kidneys, and various white adipose tissues (including epididymal, inguinal, and perirenal white fat) were weighed and collected.

2.4 Biochemical Characteristics of Liver and Blood

Cholesterol (TC), triglyceride (TG), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), alanine aminotransferase (ALT), and aspartate aminotransferase (AST) levels in serum were assessed using the Nanjing Jiancheng kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The levels of TG and TC in the liver were analyzed using the same kit. The levels of IL-1 β , TNF- α , and IL-6 in liver tissue were determined using commercial assay kits (Solarbio, Beijing, China). The concentrations of SOD, CAT, and MDA were measured using kits from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Detailed procedures are provided in Supplementary Material 1.

2.5 Glucose Metabolism

At week 12, random blood glucose was measured at 9:00 AM without fasting using a blood glucose meter (GA-3, Sinocare Inc. (Sannuo), Changsha, China). After a 12-hour fast, random fasting blood glucose and oral glucose tolerance test (OGTT) were performed. The OGTT procedure was conducted as previously outlined with slight adjustments. Following, a 12-hour fast, blood glucose levels were assessed at 0, 30, 60, 90, and 120 min-post 2 g/kg glucose administration using a blood glucose meter. The OGTT AUC was determined by calculating the area under the curve of the OGTT.

2.6 Histopathology of Liver and Adipose Tissue

Histopathological analysis of liver and adipose tissue was referred to the method of Ren et al. with minor modifications. Briefly, fresh liver and adipose tissue were fixed in 4% paraformaldehyde for 24 h. Following gradient alcohol dehydration, tissue blocks were embedded in paraffin and sectioned into 5 μ m slices using a microtome. Tissue samples underwent hematoxylin and eosin (HE) staining. Finally, sections were dehydrated in xylene-containing absolute ethanol and sealed for observation. The average size of HE-stained cells in adipose tissue was measured using Image J (National Institutes of Health, NIH, Bethesda, MD, USA).

2.7 Immunohistochemistry of Liver Tissue

Liver tissue immunohistochemistry was performed according to the method of Tliba et al. with minor modifications. Briefly, FASN and SERBEP-1c antibodies were diluted at a 1:250 ratio. Paraffin sections of

liver and kidney were stained via immunohistochemistry (IHC). Stained sections were evaluated using a standard light microscope (Olympus BX 21, Olympus Optical Co., Ltd., Japan) and photographed with a high-resolution camera (Canon®).

2.8 Determination of Fecal Lipids

The total lipid content in feces was determined according to the method of Hiel et al. with minor modifications. At the beginning of the 12th week of feeding, feces (collected within 24 h) were obtained from the cages, freeze-dried, ground, and sieved. Weighed 0.5 g of powdered feces and put into 2 mL of physiological saline solution and vortex for 1 min, added 5 mL of chloroform-methanol solution (2:1, v/v) and vortex for 1 min, then centrifuge the suspension at 6000 g for 10 min at 4°C to separate the two liquid phases. Furtherly, separated the lower organic phase and collected it into a pre-weighed glass tube. Finally, evaporated the sample in an extraction fume hood for 2-3 days; the difference in the glass tube's weight represented the total fat content of the feces. TG, TC, and fecal lipase activity in feces were determined using Nanjing Jiancheng kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

2.9 Liver Tissue Transcriptome

Liver tissue transcriptomics was performed according to the previously described method of He et al. [32] with minor modifications. Briefly, the total RNA was extracted from liver tissue using the VeZol Total RNA Extraction Kit (Nanjing Vazyme Biotech Co., Ltd., Nanjing, China). RNA concentration and integrity were assessed using a Qubit Fluorometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and a Qsep400 high-throughput biofragment analyzer (Bioptic Inc., Jiangsu, China). Library preparation was performed using the VAHTS Universal V10 RNA-seq Library Prep Kit for Illumina (Premixed version) (adapted for MGI platform) according to the manufacturer's protocol. HISAT was employed to establish the reference genome index, and paired-end clean reads were aligned against the reference genome. RNA extraction and detection, library preparation, sequencing, and bioinformatics were all completed by Wuhan Maiwei Metabolic Biotechnology Co., Ltd. (Wuhan, China).

2.10 RT-qPCR Assay

Total RNA was extracted from liver tissue using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Subsequently, cDNA was synthesized with Hifair III First Strand cDNA Synthesis SuperMix. The RT-qPCR reaction utilized Hieff-qPCR SYBR Green Premix. The primer sequence was detailed in Supplementary Table S2.

2.11 Gut Microbiota Analysis

After 12 weeks of feeding, cecal contents were collected into sterile tubes and immediately stored at -80°C. Fecal microbial DNA was extracted using the EZNA Soil DNA Kit (Omega Bio-Tek, Norcross, GA, USA) according to the manufacturer's instructions. The V4 fragment of the 16S rRNA gene was amplified using the following primers: 515F and 806R. Qualified amplicons were subjected to high-throughput sequencing on the MGI platform by Metware Biotechnology Co., Ltd. (Wuhan, China). 16S rRNA sequencing

data were analyzed using QIIME 2 for bioinformatics processing, with a focus on alpha and beta-diversity analyses to characterize microbial community structure^[33]. Differential abundance-analysis employed two complementary approaches: 1) DESeq2 (R v4.2.0), which normalized raw ASV counts generated from MGI sequencing via the median ratio method for accurate detection of taxa with differential abundance among groups; 2) LEfSe analysis on the Bioincloud platform, first screening for potential intergroup differential taxa via Kruskal-Wallis test ($P < 0.05$), followed by Linear Discriminant Analysis (LDA score >2.0) to assess biological effect size, and core biomarkers were ultimately identified with $\text{padj} < 0.05$ after Benjamin-Hochberg correction.

2.12 Statistical analysis

Data were expressed as mean $\pm$ standard deviation. Statistical analysis was performed using IBM Statistics SPSS 25.0. One-way analysis of variance (ANOVA) was conducted using LSD or Duncan's test, where $P < 0.05$ represented statistically significant differences. All significant differences were indicated in the figures and tables. Figures and tables in this study were created using GraphPad Prism (9.0) (GraphPad Software, Inc., San Diego, California, CA, USA) and Origin Pro 2021 (Origin Lab Inc., Northampton, Massachusetts, MA, USA). Additionally, *, **, and *** were denoted $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

3. Results

3.1 Phenotypic Effects of EWP in HFD-Induced Obese Mice

The experimental design in this study was shown in Fig. 1A. The weights of the mice bodies were recorded weekly (Fig. 1C), revealing significantly greater weight gain in the HFD-fed groups compared to the Control group. Mice administered EWP at different concentrations exhibited significantly lower average body weights than the Model group (Fig. 1B-1E). Notably, after 12 weeks, EWP administration significantly reduced mice body weight in a concentration-dependent manner. The EWP-1200 and EWP-1500 groups exhibited the most obvious weight loss, with no significant difference observed relative to the Orlistat positive control group ($P > 0.05$). Compared with the model group, body weight was reduced by 21.26% and 22.16% in the EWP-1200 and EWP-1500 groups, respectively, with no significant difference observed between the two groups ($P > 0.05$) (Fig. 1D and 1B). Furthermore, dynamic fluctuations were observed in overall food intake among mice (Fig. 1F). At the end of week 12, mice fed HFD consumed significantly more food than Control mice. The Model group (HFD without treatment) had the highest intake among all HFD-fed groups. However, no statistically significant differences were observed between the Model group and any EWP-treated group ($P > 0.05$). The HFD supplement also significantly increased the absolute mass of liver, kidney, spleen, epididymis, white adipose tissues (eWAT), inguinal white adipose tissue (iWAT), and perirenal adipose tissues (Fig. 1G-1M). EWP intervention dose-dependently reversed these trends, with the EWP-1500 group showing the most pronounced suppression of organ and fat mass ($P < 0.05$). However, no statistically significant differences were observed between the EWP-1500 and EWP-1200 groups ($P > 0.05$). These results

indicated that EWP significantly ameliorated HFD-induced abnormal tissue mass increases, thereby mitigating obesity-induced systemic adiposity. These further demonstrated that EWP possessed an obesity-alleviating effect with a certain degree of concentration dependence.

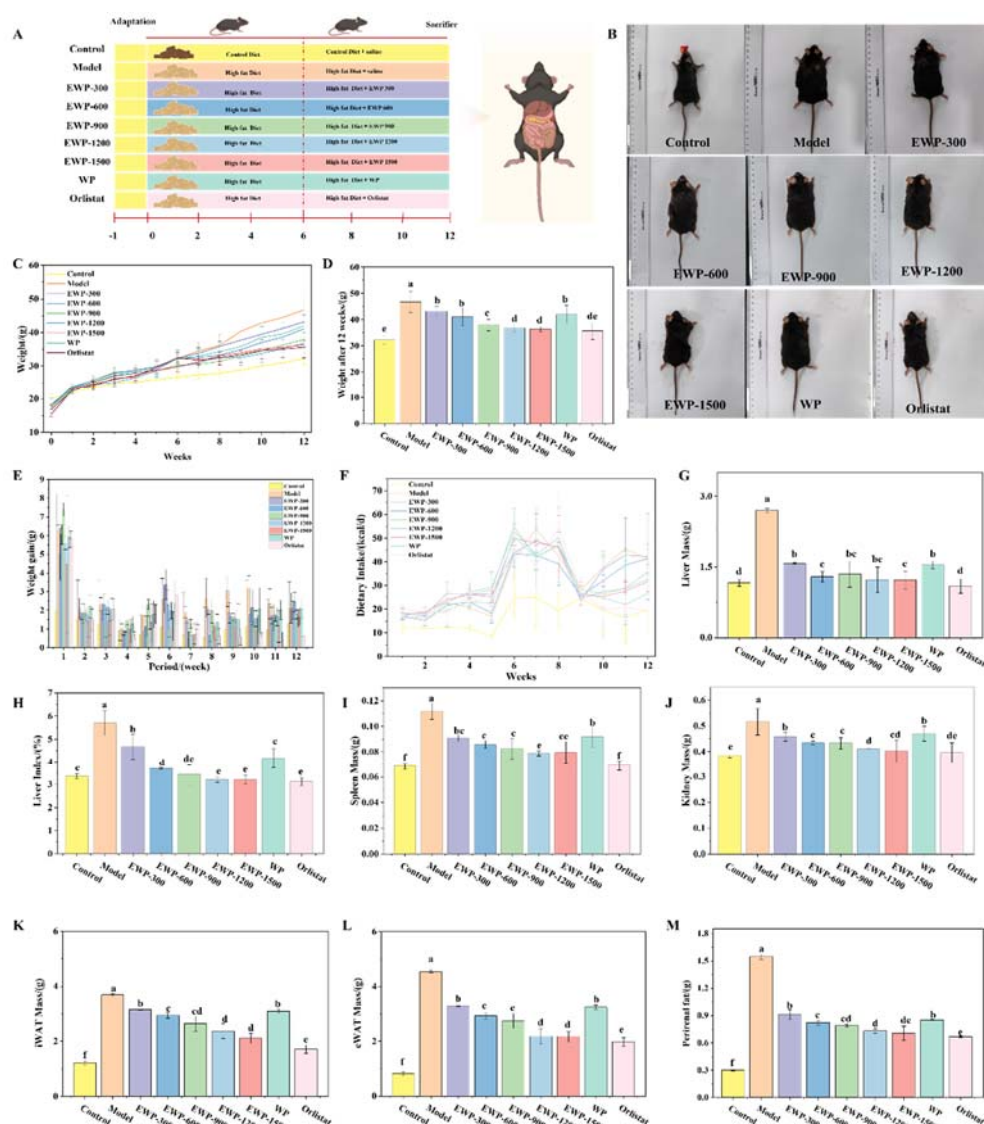


Figure 1 Effect of EWP on Phenotypic of HFD-induced obese mice

Note: A illustrated the experimental design; B showed the morphological characteristics of mice after 12 weeks of feeding; C depicted the trend in body weight changes in mice over the -12 week feeding period; D presented the final body weight of mice after 12 weeks; E displayed the weekly body weight increment changes in mice during the 12-week feeding period; F represented the daily food intake changes from week 1 to 12; G and H exhibited the liver weight and liver index; I to M showed the weights of spleen, kidney, inguinal white adipose tissue (iWAT), epididymal white adipose tissue (eWAT), and perirenal adipose tissue, respectively. Values were mean $\pm$ S.D. ($n = 8$). Lowercase letters denoted significant differences between groups, with a significance level of $P < 0.05$.

3.2 Effects of EWP on Blood Biochemical Levels in HFD Obese Mice

Figure 2 illustrated the effects of EWP on blood biochemistry in HFD-fed obese mice. Compared with the Control group, the HFD groups were exhibited significantly elevated lipid parameters (total triglycerides (TG), total cholesterol (TC), high-density lipoprotein-cholesterol (HDL-c), low-density lipoprotein-cholesterol (LDL-c)) ($P < 0.05$) (Fig. 2A-D). The Model group exhibited the highest levels of TG, TC, HDL-c, and LDL-c at 2.84, 6.89, 4.39, and 0.37 mmol/L, respectively. EWP reduced HFD-induced lipid levels in a

dose-dependent manner, no significant differences in lipid levels were observed between the EWP-1200 and EWP-1500 groups ($P > 0.05$). In comparison to the Control group, HFD-induced obese mice showed notably higher fasting plasma glucose (FPG) levels ($P < 0.05$), reaching the peak in the Model group at 6.8 mmol/L. The EWP intervention progressively reduced FPG levels, with the EWP-1500 group demonstrating the lowest level at 4.07 mmol/L ($P < 0.05$). The difference between EWP-1200 and EWP-1500 was not significant (Fig. 2E). In the OGTT test, the HFD groups exhibited significantly higher blood glucose levels ($P < 0.05$) (Fig. 2F). EWP intake significantly reduced blood glucose levels in HFD mice, which normalized after 2 h. Our study also indicated that the area under the OGTT curve (AUC) was significantly increased in the Model group (Fig. 2G). EWP intervention normalized the OGTT-AUC in HFD mice in a dose-dependent manner, with the lowest OGTT-AUC observed in the EWP-1500 group. These results demonstrated that EWP not only downregulated HFD-induced hyperglycemia but also restored impaired glucose tolerance, which was consistent with the trend of improved serum lipid levels.

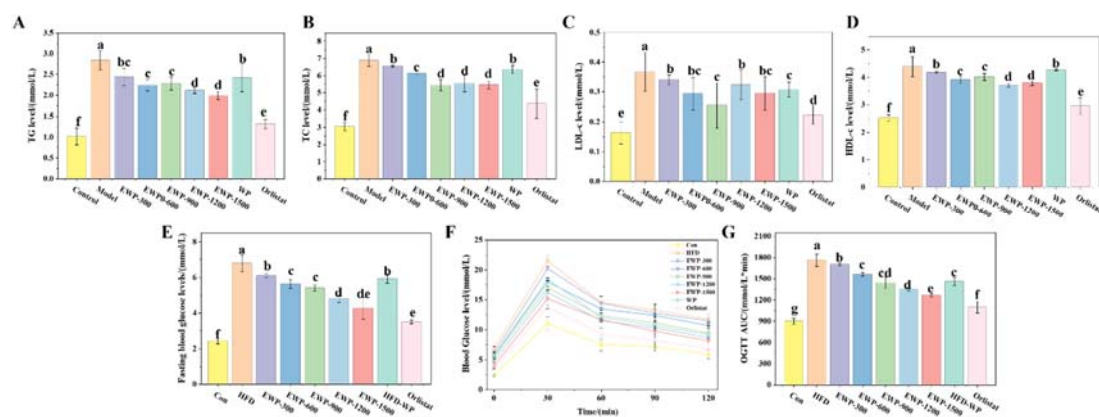


Figure 2 Effects of EWP on Blood Biochemistry in HFD-induced obese mice

Note: A-G displayed mouse serum levels of TG, TC, LDL-c, HDL-c, FBG, OGTT, and OGTT AUC, respectively. Values were mean $\pm$ S.D. ($n = 5$). Varied lowercase letters denoted significant differences among different groups ($p < 0.05$).

3.3 Effects of EWP on Fecal Lipids in HFD-induced obese mice

Total fecal lipid content in HFD-fed mice was significantly higher than that of the Control group ($P < 0.05$) (Fig. 3). However, EWP intake was promoted total fecal lipid content in HFD-fed mice. The high-dose EWP group exhibited significantly elevated fecal lipid levels, with the EWP-1200 group showing the highest content at 104.49 mg/g ($P < 0.05$). This result indicated that EWP intake effectively promoted lipid excretion in the feces of HFD mice, thereby reducing lipid accumulation in their bodies. Compared with the Control group, fecal lipase activity was significantly increased in the HFD group, while EWP intake significantly reduced fecal lipase activity in HFD mice (Fig. 3D). Furthermore, the EWP intake elevated fecal triglyceride levels and cholesterol excretion in mice fed by HFD, possibly resulting from its suppression of lipase activity and decreased lipid absorption. These results revealed that EWP effectively enhanced lipid excretion in HFD-induced obese mice, thereby alleviating obesity. Such an effect was possibly attributed to the modulation of lipid metabolism by EWP. The findings were consistent with the observed changes in blood lipid levels.

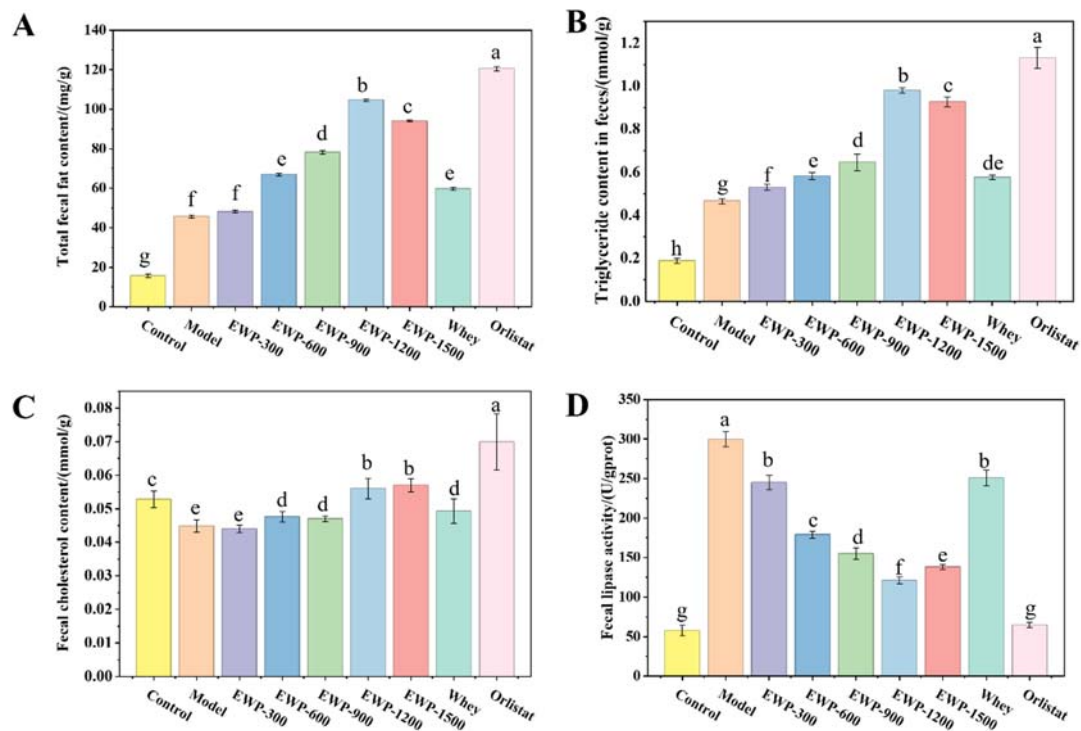


Figure 3 Effect of EWP on lipid digestion characteristics in HFD-induced obese mice

Note: A-D presented total fecal fat, triglycerides, cholesterol content, and fecal lipase activity, respectively. Values were mean $\pm$ S.D. (n=5). Lowercase letters indicated significant differences among different groups ($P < 0.05$).

3.4 Effects of EWP on Adipose Tissue Pathology in HFD Obese Mice

Histopathological analysis revealed that prolonged HFD intake led to enlarged epididymal adipose tissue (eWAT) cells, disorganized adipocyte arrangement, and severe lipid accumulation, indicating adipocyte hypertrophy (Fig. 4). This adipocyte hypertrophy was most pronounced in the Model group, where the average cell size increased to $18,798.18 \mu\text{m}^2$. Administering EWP significantly improved adipocyte hypertrophy with a dose-dependent effect. EWP-1200 and EWP-1500 exhibited the most potent reversal of adipocyte hypertrophy, reducing mean cell size to 5849.16 and $6022.14 \mu\text{m}^2$, respectively. Interestingly, the Orlistat group also significantly reversed the adipocyte size, showing no significant difference in the Control group. This further demonstrated that EWP intake effectively inhibited adipose tissue cell hypertrophy and reduced lipid accumulation.

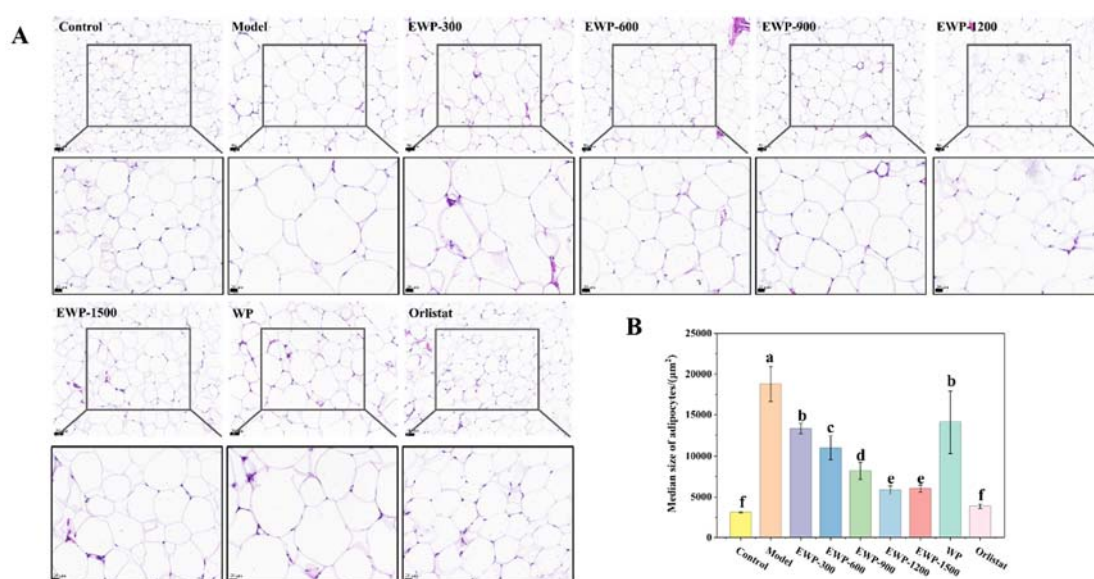


Figure 4 Effect of EWP on the histopathology of eWAT tissue in HFD-induced obese mice

Note: A and B presented the histopathological images of eWAT tissues (H&E, ×20 and ×40) and illustrated the size of cells within the adipose tissue of each group, respectively (n=4). The images containing boxes and the corresponding images below the boxes represented the tissue field maps of 50 μm and 20 μm in each group, respectively.

3.5 Effects of EWP on liver histopathology and lipid metabolism

Long-term HFD administration induced morphological alterations in mice hepatocytes, which were characterized by enlarged intercellular spaces, loose texture, and predominant edema, resulting in overall structural disorganization (Fig. 5A). Furthermore, the Model group exhibited numerous hepatocytes containing clearly visible lipid droplets, indicating abnormal lipid metabolism within hepatocytes. More importantly, AST and ALT activities in the Model group were elevated 1.24-fold and 5.61-fold, respectively, compared to the Control group (Fig. 5D and 5E). EWP supplementation significantly decreased AST and ALT activities. The EWP-1200 and EWP-1500 groups exhibited the most prominent ameliorative effects, with no significant difference observed between the two groups ($P > 0.05$). Additionally, we found the EWP intake significantly reduced the levels of anti-inflammatory factors (IL-1 β , TNF- α , IL-6) in the livers of HFD-induced obese mice. It also reversed the activity of key oxidative stress enzymes (CAT and SOD) and increased liver MDA levels. The specific data was presented in Supplementary Fig. S1. Notably, the measurement of hepatic triglyceride levels in HFD-obese mice (Fig. 5B) revealed significantly higher triglyceride levels in the model group compared to the Control group. Supplementation with EWP significantly reduced triglyceride (TG) levels in the livers of HFD-induced obese mice ($P < 0.05$). Specifically, the EWP-1200 group exhibited the lowest TG content at 0.25 mmol/g. Concurrently, the EWP-1500 group exhibited the lowest total cholesterol (TC) content at 0.014 mmol/g (Fig. 5C). However, statistical analysis revealed no significant differences in hepatic TG and TC levels between the EWP-1200 and EWP-1500 groups ($P > 0.05$). These results indicated that prolonged HFD also induced hepatic steatosis. However, EWP supplementation resulted in reduced lipid accumulation, diminished liver injury, and hepatocyte morphology approaching normal. It further demonstrated that EWP's beneficial effects on HFD-obese mice included mitigating hepatic degeneration. These findings further proved that a prolonged high-fat diet induced liver injury and increased

hepatic lipid accumulation, while EWP intake effectively reversed high-fat-induced liver damage and lipid accumulation in a concentration-dependent manner. These findings were consistent with EWP's lipid-lowering effects.

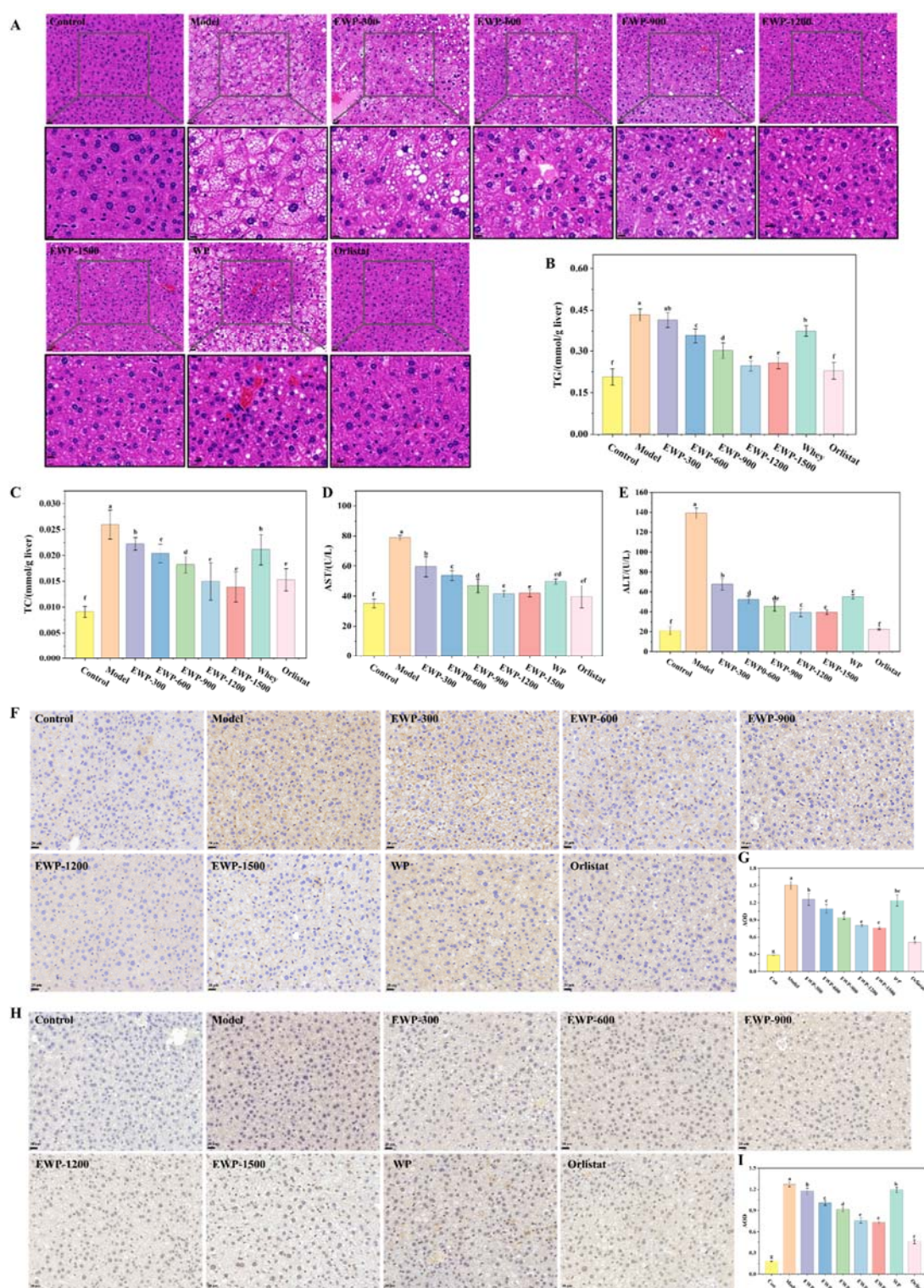


Figure 5 Effects of EWP on Liver Histopathology and Immunohistochemistry

Note: Figures A to E presented representative micrographs (H&E, ×40 and ×100) of liver tissue slices from each group (n=4), along with measurements of liver triglyceride (TG) levels, cholesterol (TC) levels, and liver function, which included the activities of key enzymes ALT and AST (n=5). Figure A included a box, beneath which the corresponding images displayed tissue field maps for each group at 20 μm and 10 μm, respectively. Figures F and G illustrated the liver immunohistochemistry map (×40) and the area of optical density (AOD) map for SREBP-1c expression, respectively (n=4). Figures H and I depicted the liver immunohistochemistry map (×40) and the AOD map for FASN expression, respectively.

Immunohistochemical analysis (Fig. 5F-I) revealed that SREBP-1c and FASN positive signals were weak in the liver tissue of the Con group; while the Model group exhibited significantly elevated SREBP-1c and FASN positive signals and expression levels ($P < 0.05$), driving excessive lipid synthesis. In the EWP intervention groups, both SREBP-1c and FASN positive signals diminished with increasing dose, with medium-to-high doses (e.g., EWP-1200, 1500) significantly downregulating their expression ($P < 0.05$). The WP group exhibited higher SREBP-1c and FASN expression than that of the EWP-600 group, indicating that EWP exerted a more pronounced and precise inhibitory effect on key lipid synthesis factors.

3.6 Effects of EWP on the Liver Transcriptome of HFD-induced obese mice

Transcriptomics was used to reveal the molecular mechanism of EWP relieving lipid transcriptional modulation in HFD obese mice. Orthogonal partial least squares discriminant analysis (OPLS-DA) assessed the quality and variability of liver gene characteristics in mice from the Control group, Model group, and EWP group (Fig. 6A). The analysis indicated that liver samples from different groups were clearly separated and significantly clustered, demonstrating substantial differences among these groups. The Venn diagram (Fig. 6B) revealed 633 differentially expressed genes (DEGs) using the criteria $FDR < 0.05$ and $|\log_2 \text{fold change (FC)}| \geq 1$, mostly from comparisons between EWP-treated groups and the HFD model group. Notably, EWP significantly regulated the global hepatic gene expression profile in HFD-induced obese mice, as confirmed by principal component analysis (PCA) and hierarchical clustering heatmap (Figs. 6D–6F). Specifically, relative to the Control group, the Model group had 157 downregulated and 407 upregulated genes (Figs. 6D and 6G), while the EWP group, displayed 84 upregulated and 59 downregulated genes (Figs. 6E and 6J). Compared to the Model group, the EWP group had 387 downregulated genes and 112 upregulated genes (Fig. 6F and 6M). Based on the expression of differentially regulated genes across groups, KEGG analysis was performed, focusing on the KEGG pathways of genes differing between EWP_VS_Control (Fig. 6H-I) and EWP_VS_Model (Fig. 6K-L) groups. KEGG analysis revealed that differentially regulated genes in the EWP_VS_Control group were predominantly clustered in pathways, including MAPK signaling, Ras signaling, Rap1 signaling, AMPK signaling, and insulin signaling (Fig. 6H). While in the EWP_VS_Model group, differentially expressed genes were predominantly clustered in the PPAR signaling pathway, MAPK signaling pathway, AMPK signaling pathway, adipokine signaling pathway, and insulin signaling pathway (Fig. 6K). Among these, the PPAR signaling pathway played a dominant role in regulating lipid metabolism, with enriched differentially expressed genes including *Hmgcs2*, *Lpl*, *Apoa5*, *Plin2*, *Acox2*, *Cd36*, *Acs11*, *Pparg*, *Plin4*, and *Ppara* (Fig. 6L). Furthermore, GO analysis (Fig. 6N-O) explored the cellular functions regulated by differentially expressed genes in the EWP and Model groups. These genes were enriched across three categories: biological processes, cellular components, and molecular functions. Notably, most differentially expressed genes were concentrated in lipid-related biological processes, including lipid metabolism, lipid transport, lipid biosynthesis, lipid storage, glycolipid metabolism, and lipid entry into cells. These altered biological processes indicated that EWP supplementation could mitigate HFD-induced obesity by regulating biological processes. To validate the transcriptomic findings, seven key lipid metabolism genes with the

highest differential expression in the EWP_VS_Model group were selected for RT-qPCR analysis (shown in Supplementary Fig. S2), including CD-36, FASN, SERBP-1c, PPAR α , PPAR γ , HSL, and ACC. Results showed that compared to the Control group, the Model group exhibited significantly increased relative expression of genes, including CD-36, FASN, ACC, SERBP-1c, and PPAR γ , while genes such as PPAR α and HSL showed significantly decreased relative expression. However, the expression trends of these genes were significantly reversed in the EWP group ($P < 0.05$).

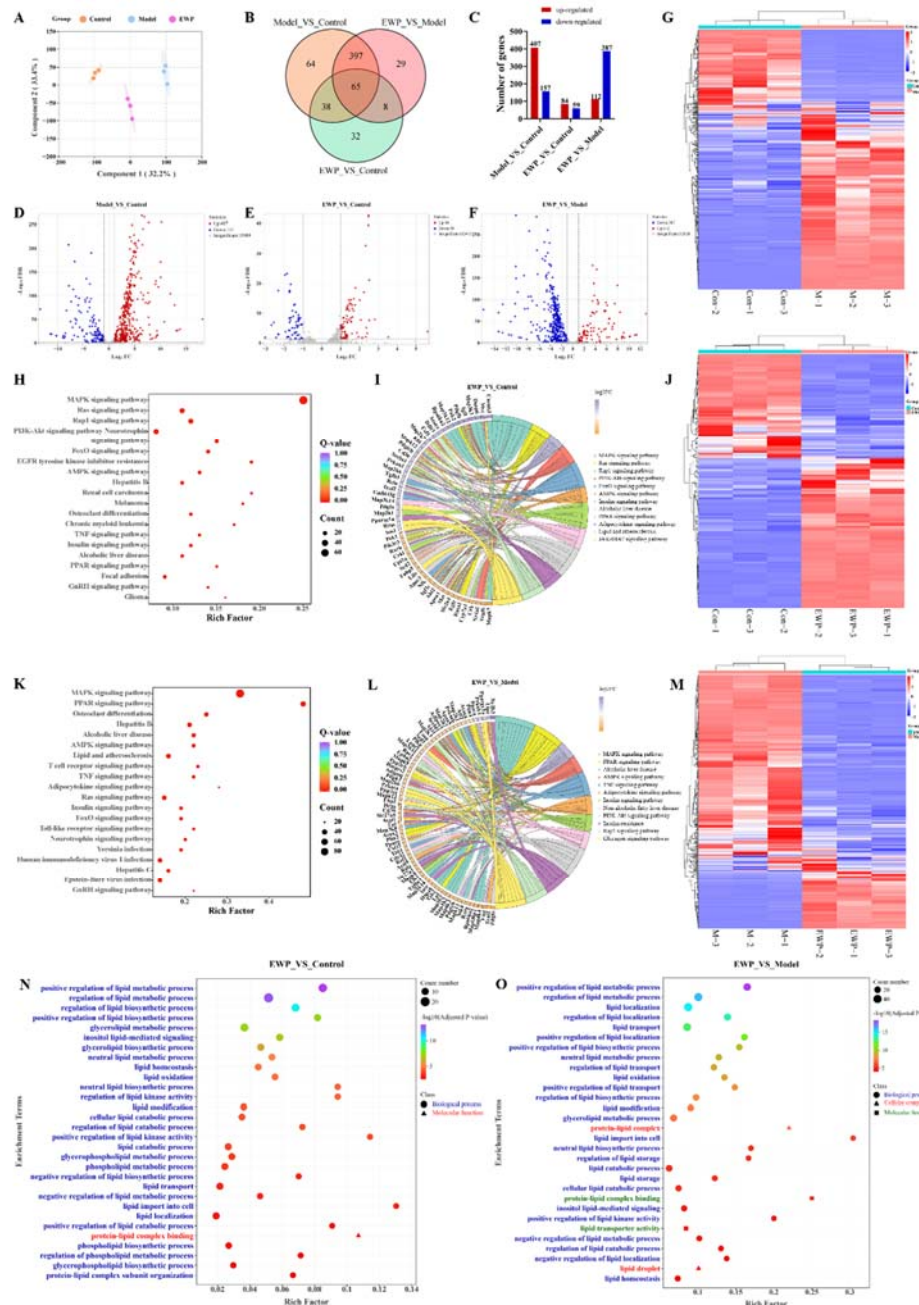


Figure 6 Illustrated the impact of EWP on liver transcriptomics in HFD-induced obese mice

Note: A: OPLS-DA analysis; B: Venn diagram illustrating differentially expressed genes (DEGs) across the groups; C: Number of upregulated and downregulated DEGs in comparisons between two groups. D-F: Volcano plots of DEGs for the Model_VS_Control group, EWP_VS_Control group, and EWP_VS_Model group, respectively; G, J, and M: Cluster heatmaps of DEGs for the Model_VS_Control group, EWP_VS_Control group, and EWP_VS_Model group, respectively; H-I: KEGG analysis of differentially expressed genes between EWP_VS_Control groups and string plots of DEGs in related signaling pathways; K-L: KEGG analysis of intergroup DEGs and string plots of DEGs associated with signaling pathways in EWP_VS_Model; N and O: GO enrichment pathways for the EWP_vs_Control group and the EWP_vs_Model group, respectively. The result values of RNA seq were expressed as the mean $\pm$ SEM of 3 biological replicates.

3.7 Effects of EWP on Gut Microbiota in HFD-induced obese mice

16S rRNA sequencing was employed to investigate how EWP alleviated obesity progression and development by modulating the gut microbiota. Venn diagram analysis (Fig. 7A) revealed that the Control group exhibited the highest number of ASVs, followed by the EWP group, while the Model group showing the lowest abundance. α -diversity analysis, commonly used to assess changes in microbial community diversity (Fig. 7B-D), primarily included richness (Chao1 and ACE) and evenness (Shannon). The Control group exhibited the highest richness and evenness, followed by the EWP group, while the Model group showed the lowest values. After FDR correction for multiple testing, these differences in α -diversity indices among the three groups were statistically significant ($\text{padj} < 0.05$). These results indicated that the HFD reduced microbial richness and evenness, while EWP partially restored these parameters. These findings were consistent with previous studies demonstrating rice protein's regulation of obesity-related gut microbiota [34]. Principal coordinate analysis (PCoA) based on Weighted UniFrac and Bray-Curtis distances (Figs. 7D and 7E) revealed clear β -diversity separation among the Control, Model, and EWP groups, with tight intra-group clustering (Figs. 7F and 7G). PERMANOVA analysis based on these two distance matrices, followed by FDR correction, confirmed that the inter-group separation was statistically significant ($\text{padj} < 0.05$), indicating that HFD-induced obesity altered gut microbial community structure, which was partially reversed by EWP intervention.

Based on PCoA results, further analysis at the phylum level revealed compositional patterns (Fig. 7I). Across all samples, *Bacteroidetes* and *Firmicutes* were the two most dominant phyla, accounting for $34.09\% \pm 0.04\%$ and $60.45\% \pm 2.78\%$ of the Control group, respectively. Compared to the Control group, the Model group showed a significant increase in *Firmicutes* and *Desulfobacterota*, a significant decrease in *Bacteroidetes*, and an increased *Firmicutes/Bacteroidetes* (F/B) ratio (to 3.97 ± 0.31). EWP treatment reduced the F/B ratio by significantly decreasing *Firmicutes* and increasing *Bacteroidetes* abundance. Notably, EWP intake also significantly increased *Verrucomicrobia* abundance.

At the genus level, compared to the Control group, the HFD group showed significantly reduced *Lactobacillus* and *Bifidobacterium* abundances, while *Lachnoclostridium* and *Mucispirillum* abundances increased. Compared to the Model group, EWP intake significantly increased the abundance of the genera *Akkermansia*, *Bifidobacterium*, and *Bacteroides*. Based on this, nonparametric Kruskal-Wallis (KW) and rank-based LEfSe comparisons ($\text{LDA} > 2.0$, $\text{padj} < 0.05$) were used to identify bacterial groups with significant abundance differences across groups. Comprehensive differential analysis across five taxonomic levels from phylum to genus identified 28 significantly different microbial communities across the three groups: 12 unique to the Model group, 5 unique to the EWP group, and 11 unique to the Control group (Fig. 7L). EWP intake enriched communities, including *Akkermansiaceae*, *Verrucomicrobiota*, *Akkermansia*, and *Butyrivicoccus*. Bacterial groups such as *Erysipelotrichales*, *Anaerotruncus*, and *Blautia*, recognized as biomarkers of obesity [35-37], showed increased relative abundance in obese mice. However, EWP intake reduced the abundance of these bacteria. To further clarify the degree of microbial differences between the two groups, LEfSe analysis

was performed with FDR correction ($LDA > 2.0$, $padj < 0.05$). Comparing the Model and EWP groups identified 44 significantly different taxa, with LEfSe analysis highlighting 30 significant differences enriched in the Model group. *Firmicutes*, *Clostridia*, *Anaerotruncus*, *Bacilli*, *Erysipelotrichaceae*, and *Oscillibacter* were identified as biomarkers for the Model group [38].

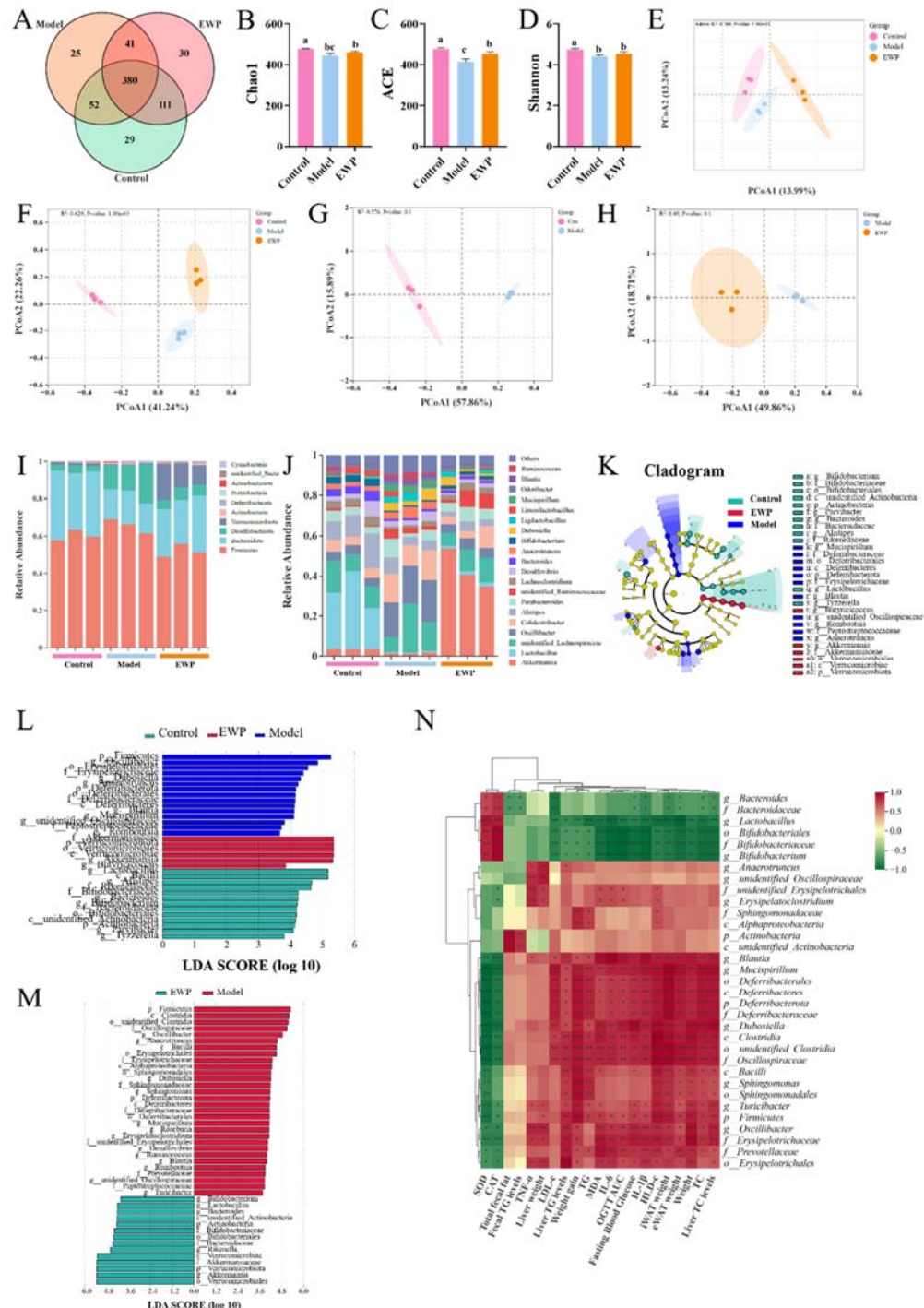


Figure 7 Effect of EWP on gut microbiota of HFD-induced obese mice

Note: A showed the Venn diagram of ASVs; B-D demonstrated the Chao1, ACE, and Shannon indices, respectively; D was the PCoA analysis between three groups based on the weighted_unifrac algorithm; F-H displayed PCoA analysis based on t-Bray-Curtis algorithm for three groups, Control and Model group, and EWP and Model group, respectively; I and J were the microbial community species at the phylum and genus levels, respectively; K-L represented the inter group LEfSe analysis Cladogram and bar chart, respectively; M was the differential microbial community map of LEfSe analysis between EWP and Model groups; N was the clustering heatmap of relevant differential bacterial genera and obesity phenotypes; *, **, and *** denoted $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively. The resulting values of 16S rRNA were expressed as the mean of 3 biological replicates $\pm$ SEM.

Based on the above LEfSe analysis results, Spearman correlation analysis with FDR correction was further performed to clarify the potential relationship between HFD-induced obesity-related phenotypic parameters and gut microbiota (Fig. 7N). The study identified *Blautia*, *Mucispirillum*, *Dubosiella*, *Sphingomonas*, *Turicibacter*, and *Oscillibacter* as the primary bacterial genera that were related to the HFD, and these genera showed significantly positive correlations with obesity-related phenotypic parameters. Crucially, *Anaerotruncus*, *Erysipelatoclostridium*, *unidentified_Oscillospiraceae*, and *unidentified_Erysipelotrichales* showed significantly positive correlations with liver weight and TNF- α . Concurrently, *Erysipelatoclostridium* and *unidentified_Oscillospiraceae* also exhibited significantly positive correlations with MDA, IL-6, and OGTT AUC. However, *Bacteroides*, *Lactobacillus*, and *Bifidobacterium* showed significantly negative correlations with serum and hepatic TC, iWAT, eWAT, fasting blood glucose, and IL-1 β . Collectively, these correlation analysis results suggested that EWP intake might improve HFD-induced obesity-related metabolic disorders by modulating the composition of specific gut microbiota—specifically, by reducing the abundance of pro-obesogenic genera (e.g., *Oscillibacter*, *Erysipelatoclostridium*) and increasing the abundance of beneficial genera (e.g., *Bacteroides*, *Lactobacillus*, *Bifidobacterium*).

4. Discussion

Long-term HFD consumption could cause weight gain and increased organ mass, leading to systemic obesity and subsequent metabolic disorders (e.g., hyperglycemia, hyperlipidemia)^[13, 15]. Our study showed that EWP significantly reduced body weight in HFD-obese mice, with the EWP-1200 and EWP-1500 groups exhibiting the strongest effects. At equivalent doses, EWP was significantly more effective than WP in reducing weight. Histopathology revealed adipocyte hypertrophy and severe hepatic vacuolation in the HFD group, while EWP ameliorated tissue damage, reduced serum lipid levels in a concentration-dependent manner, and enhanced fecal lipid excretion. Given the gut's key role in lipid homeostasis and lipase-mediated intestinal lipid digestion, EWP likely exerted anti-obesity effects by regulating lipid metabolism to inhibit intestinal lipid absorption and promote excretion. This aligned with previous findings that dietary EW and EWH reduced hepatic (ectopic) fat accumulation in HFD-induced rats by inhibiting fat absorption and delivering fat to the liver^[39]. Since current studies are largely limited to lipid absorption and excretion, future fat balance assessments will be performed to confirm the regulatory effects of EWP on lipid metabolism.

As the central lipid metabolic organ, the liver is critical for maintaining lipid homeostasis and fully participates in host lipid metabolism^[40]. HFD-fed mice exhibited enlarged hepatic intercellular spaces, loose tissue texture, and increased lipid droplets (Fig. 5A). EWP effectively mitigated HFD-induced elevations in AST/ALT (Fig. 5D-5E), decreased hepatic TG (Fig. 5B), and reversed liver damage, likely via regulating hepatic lipid metabolism in HFD-obese mice. SREBP-1c, a key transcription factor activating hepatic triglyceride/fatty acid synthesis genes^[41], induced FASN to regulate fatty acid synthesis^[16]. Immunohistochemistry showed that EWP significantly downregulated hepatic SREBP-1c and FASN expression in HFD-obese mice (Fig. 5F-5I), indicating that EWP mitigates HFD-induced obesity by modulating hepatic lipid metabolism, which correlated with improved hepatic metabolic function.

To elucidate EWP's anti-obesity mechanism, liver transcriptome analysis (Fig. 8) confirmed that EWP acted by regulating hepatic lipid metabolism, closely linked to restored liver metabolic function. The PPAR signaling pathway, a key obesity regulator, modulated lipid metabolism-related gene expression [42] and was regulated by the AMPK pathway [43, 44]. AMPK regulated lipid metabolism by inhibiting the PPAR pathway (including SREBP-1c) and activating Hormone-Sensitive Lipase (HSL) [45, 46], suggesting EWP alleviated obesity by modulating PPAR pathway-related proteins to precisely regulate lipid metabolism.

Critical comparison of the literature revealed that while the anti-obesity effects of egg white protein (EWP) have been reported, most prior studies focused on a single gene in the PPAR pathway and lacked systematic exploration of its regulatory networks. For instance, egg white protein hydrolysate reduced PPAR γ 2 abundance and promoted insulin-induced Akt phosphorylation, thereby shrinking adipocytes in HFD-induced obese rats [47]. Other related studies have shown that ovalbumin hydrolysate improves glucose tolerance and insulin signaling in HFD-fed mice [48]. IRW enhances glucose tolerance via skeletal muscle insulin/AMPK activation in HFD-fed C57BL/6 mice [49], and egg white protein derivatives reduce hepatic triglyceride (TG) levels and lipid droplet size through AMPK phosphorylation and microsomal triglyceride transfer protein upregulation [50]. Notably, none of these studies explored the entire PPAR pathway or its crosstalk with other metabolic pathways—a key distinction of our work. We systematically investigated EWP's regulation of the PPAR pathway and its association with hepatic lipid metabolism, addressing the gap in current research regarding the systematic mechanistic exploration of EWP's anti-obesity effects.

Notably, while WP exerted anti-obesity effects in HFD models, our study found EWP was superior to WP at equivalent doses, likely due to distinct pathways. WP reduced hepatic TG via the AMPK/mTOR pathway [51]; bovine α -lactalbumin/hydrolysate improved hepatic fat accumulation by PPAR γ downregulation and PPAR α upregulation [52, 53], though the underlying signaling pathways were not further explored. Its derivatives exert anti-obesity effects via the IKK/MAPK signaling pathways [54], and WP promoted adipose browning to downregulate adipogenic gene expression [55]. However, most WP studies focused on glucose metabolism signaling, with few on systematic PPAR pathway regulation and hepatic lipid metabolism crosstalk. Casein reduced weight gain/hepatic lipid accumulation via intestinal lipid absorption regulation [56, 57] but had weak PPAR pathway effects and lacked EWP's hepatic lipid metabolism-gut microbiota synergistic regulation. In contrast, EWP acted via a synergistic hepatic PPAR-AMPK pathway and lipid absorption/excretion regulation—a unique advantage over WP, emphasizing our study's innovation. Gut microbiota significantly affected host health, and obesity correlated with reduced diversity and altered composition [58–60]. EWP restored gut microbial richness/evenness and normalized genera such as *Bifidobacterium*, *Lactobacillus*, *Akkermansia*, and *Bacteroidetes* in HFD mice. Correlation analysis linked EWP's microbiota modulation to improved obesity phenotypes. Fecal microbiota transplantation studies were needed to validate specific microbiota-host lipid metabolism relationships.

To further elucidate the mechanism underlying the anti-obesity effects of EWP, subsequent studies will verify our transcriptome data by Western blotting, focusing on the expression and phosphorylation of key

proteins, including PPAR α , SREBP-1c, and FASN. Protein localization will be determined by immunofluorescence, protein–protein interactions by co-immunoprecipitation (Co-IP), and protein function via gene silencing and overexpression. Combined in vivo and in vitro models will be used to characterize the molecular network of lipid metabolism regulated by EWP, supporting its potential use in obesity intervention. Since current studies are largely limited to intestinal lipid absorption and excretion, future fat balance assessments will be performed to confirm the regulatory effects of EWP on lipid metabolism.

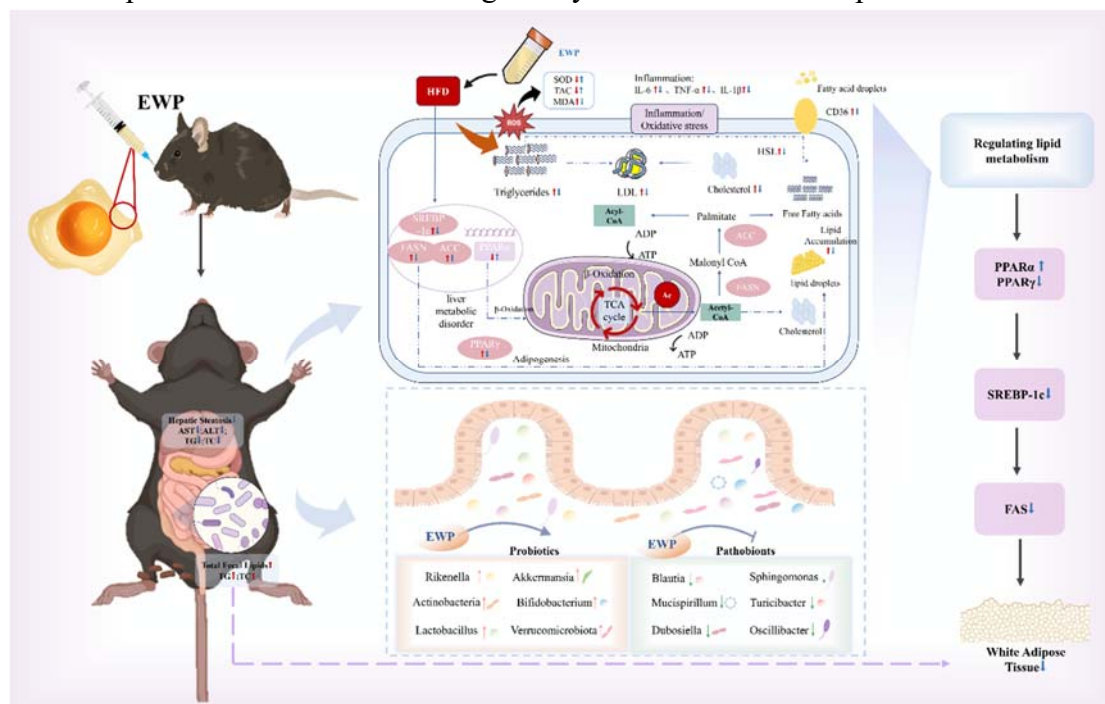


Figure 8 Potential mechanism of EWP in alleviating HFD-induced obese mice

5. Conclusion

In conclusion, the study demonstrated that EWP alleviated obesity characteristics in HFD mice in a dose-dependent manner, with EWP-1200 and EWP-1500 exhibiting optimal effects. Based on these foundational results, we explored the potential mechanisms underlying EWP's anti-obesity effects through liver transcriptomics and gut microbiota analysis. It was found that EWP intake significantly altered HFD-induced mRNA expression changes, predominantly concentrated in key pathways such as lipid synthesis and lipid metabolism. Furthermore, EWP intake might also improve lipid transcriptional modulation by enhancing HFD-induced gut microbiota composition, increasing not only the abundance of the *Bacteroidetes* phylum but also beneficial bacterial genera, including *Bifidobacteria*, *Lactobacillus*, and *Akkermansia*. These results demonstrate that EWP is a promising dietary protein candidate for obesity management. Future research should further elucidate additional molecular mechanisms responsible for its anti-obesity activity.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data Availability

Data are available upon request.

Compliance with Ethics Requirements

All procedures involving animals and their care follow institutional guidelines set by the Animal Ethics Committee of Hefei University of Technology (**No. HFUT20240405004**) and are in accordance with national and international laws and policies.

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